CARRIAGE OF STAPHYLOCOCCAL TOXIN GENES AND STRAIN TYPING OF ISOLATES FROM DOGS AND HORSES

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2018

Major Subject: Veterinary Pathobiology

ABSTRACT

Staphylococcus spp. are important causes of skin and systemic infections in people, dogs, and horses. In dogs, *S. pseudintermedius* is the most common cause of pyoderma. *Staphylococcus aureus* exfoliative toxin (ET) genes are associated with blistering skin lesions, and four purported exfoliative toxin genes have been reported in *S. pseudintermedius*: *speta, siet, expA,* and *expB.* Few studies have determined the prevalence of *S. pseudintermedius* ET genes in a large collection of canine isolates, and none have induced native ET proteins or determined if dogs produce anti-ET antibodies. The prevalence of *speta, siet, expA,* and *expB* in a collection of 500 clinical isolates from healthy and diseased dogs was 100, 99, 13, and 16%, respectively. A higher proportion of the dogs with pyoderma cultured *expA*-positive isolates when compared to healthy and diseased dogs. Native induction of EXPA and EXPB required incubation of liquid cultures with 10% CO_2, and both proteins were excreted in the culture supernatant. The SIET protein was not detected under these conditions. Canine sera contained antibodies against EXPA and EXPB. Whole genome sequencing was performed on a subset of 13 isolates. In the genomic sequences we obtained, the *expA* gene was consistently carried adjacent to a reverse transcriptase (Group II intron) and *expB* next to mobile element proteins that may facilitate horizontal transfer of the genes.

Staphylococcus aureus is associated with soft tissue and respiratory infections in horses. No studies have examined toxin and antimicrobial resistance characteristics of equine methicillin-sensitive (MSSA) and resistant (MRSA) isolates from the southern

United States (US). The MRSA prevalence in a collection of 71 clinical isolates from horses and donkeys was 19%, with most being clonal complex 8. The most common MSSA was ST1, and 66% of all isolates carried the equine-adapted leucocidin PQ, the first instance of carriage outside of Europe. The toxin and resistance genes carried were diverse and included a new prophage-encoded variant of toxic shock syndrome toxin and one mupirocin and lincosamide-resistant isolate that has never been reported in a horse. We found that MSSA in addition to MRSA carry diverse virulence factors and can be pathogenic in horses.

ACKNOWLEDGEMENTS

I would like to thank my committee chairs, Dr. Sara Lawhon and Dr. Magnus Höök, and my committee members, Dr. Rodrigues Hoffmann, Dr. Kier, and Dr. Patterson, for their support throughout the course of my PhD research. I also wish to thank the Pathology and Clinical Microbiology faculty and staff at Texas A&M University for their patience and willingness to help me. Finally, thank you to my parents, Bob and Marilyn Bryan, and to my boyfriend Benedikt S. Harrison for helping me through.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of advisor Associate Professor Sara D. Lawhon, Professor Ann Kier, and Assistant Professor Aline Rodrigues Hoffmann of the Department of Veterinary Pathobiology, Assistant Clinical Professor Adam Patterson of the Department of Small Animal Clinical Sciences, and advisor Professor Magnus Höök of the Texas A&M Health Science Center Institute of Biosciences and Technology Center for Infectious and Inflammatory Diseases. The statistical analyses for the prevalence study described in Chapter III were conducted by Professor Noah Cohen of the Department of Large Animal Clinical Sciences. Expression and purification of the recombinant *S. pseudintermedius* ExpA and ExpB (Set 2) proteins in Chapter IV were done by Assistant Research Scientist Wen Liu of the Texas A&M Health Science Center Institute of Biosciences and Technology Center for Infectious and Inflammatory Diseases. Library preparation, DNA quality control, and Illumina MiSeq sequencing of the bacterial isolates detailed in Chapters V and VI were done by Research Assistant Professor Andrew Hillhouse of the Department of Veterinary Pathobiology in the Molecular Genomics Workspace. Gross images for lesions in Chapter VI were provided by Professor John F. Edwards and Clinical Associate Professor Brian F. Porter of the Department of Veterinary Pathobiology; and Dr. Franklin Lopez of the Texas A&M Veterinary Medical Diagnostic Laboratory. All other work conducted for the dissertation was completed by the student independently.

Funding sources

Graduate study was supported by a National Institutes of Health Training Grant (5 T32 0D 011083) and departmental funding from Veterinary Pathobiology at Texas A&M University. This work was made possible in part by a Clinician Scientist Award from the American Kennel Club and a grants from the Morris Animal Foundation (D15CA-833) and the Veterinary Orthopedic Society (M1401840). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the American Kennel Club, the Morris Animal Foundation, or the Veterinary Orthopedic Society. Funding for the sequencing of the equine *S. aureus* isolates described in Chapter VI was provided by the Equine Infectious Disease Laboratory (Professor Noah Cohen, director), the Texas A&M Veterinary Medical Teaching Hospital, the Department of Veterinary Pathobiology, and laboratory funds of Associate Professor Sara D. Lawhon.

NOMENCLATURE

ACME	Arginine catabolic mobile element
EDIN	Epidermal cell differentiation inhibitor
ET	Exfoliative toxin
FFPE	Formalin-fixed paraffin embedded
IEC	Immune evasion cluster
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MLST	Multi locus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSP	Methicillin-resistant *Staphylococcus pseudintermedius*
MSSA	Methicillin sensitive *Staphylococcus aureus*
MSSP	Methicillin sensitive *Staphylococcus pseudintermedius*
PI	Pathogenicity island
SAgs	Superantigens
SCC*mec*	Staphylococcal cassette chromosome *mec*
ST	Sequence type
VMTH	Veterinary Medical Teaching Hospital
WGS	Whole genome sequencing

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CHAPTER I

INTRODUCTION

Background

Little is known about the carriage prevalence of *S. pseudintermedius* exfoliative toxin (ET) genes, if the genes can be horizontally transferred, or if carriage of ET genes is associated with patient demographics, predominate strain types or specific antimicrobial susceptibility profiles. Likewise, expression of native ET in *S. pseudintermedius* isolates with different ET carriage profiles has not been investigated. The complete toxin gene carriage profiles, strain type, and antimicrobial susceptibilities of equine *S. aureus* isolates collected from the southern United States (US) have also not been studied. To address these questions, the dissertation is divided into four aims, each comprising a chapter of the dissertation.

Specific Aims

AIM 1: Determine the prevalence of exfoliative toxin (ET) gene carriage in *Staphylococcus pseudintermedius* isolated from dogs. Determine if there are correlations between ET gene carriage, patient demographics, infection site, or methicillin resistance status. Determine if ET gene carriage is correlated to inflammation severity in canine skin biopsies.

AIM 2: Induce native expression of the EXPA and EXPB toxin proteins in bacterial isolates *in vitro*. Determine if dogs with and without a history of pyoderma or culture of *S. pseudintermedius* produce antibodies to the ET *expA* and *expB*.

AIM 3: Perform whole genome sequencing (Illumina MiSeq and Oxford Nanopore) on select *Staphylococcus pseudintermedius* isolates carrying specific ET genes to map the positions of the ET genes within the chromosome and determine if ET genes are integrated on mobile genetic elements (MGE).

AIM 4: Perform whole genome sequencing (Illumina MiSeq) on *Staphylococcus aureus* isolates from horses admitted to the Veterinary Medical Teaching Hospital. Determine the prevalence of toxin gene carriage in the isolates and if there are correlations between toxin gene carriage, patient demographics, infection site, or methicillin resistance status.

CHAPTER II

LITERATURE REVIEW

Overview of coagulase-positive Staphylococcus spp.

Staphylococcus spp. are Gram-positive coccoid bacteria that can be broadly categorized into coagulase-positive and negative groups. The eight known coagulase positive *Staphylococcus* species include: *S. aureus, S. schleiferi* subsp. *coagulans*, *S. hyicus*, *S. agnetis*, *S. lutrae*, and the *S. intermedius* group (SIG) [1,2]. Of these, *S. aureus* and SIG are the most frequently encountered in dogs and horses [1]. *Staphylococcus aureus* is an important cause of opportunistic bacterial infections in humans and animals within hospital settings and the community at large, with antimicrobial drug-resistant strains representing a significant public health hazard [3]. The SIG species are highly related to each other and comprise four separate entities: *S. intermedius*, *S. pseudintermedius*, and *S. delphini* groups A and B [2]. All of the SIG species were once classified as *S. intermedius*, which was discovered in 1976 and was often isolated from pigeons, dogs, mink, and horses as part of the native skin microbiota or as a cause of infections in dogs and cats [4,5]. *Staphylococcus intermedius* as currently defined is most frequently isolated from pigeons, and *S. delphini* has been isolated from the normal skin microbiota of dolphins, pigeons, mink, and horses [1]. *Staphylococcus pseudintermedius* was determined to be a species unique from *S. intermedius* in 2005 and is a frequent colonizer of healthy and diseased canine skin [2]. *Staphylococcus pseudintermedius* is the most common cause of pyoderma in dogs, with sporadic infections also reported in

immunocompromised people [6]. Members of the SIG can be difficult to tell apart from each other and *S. aureus* based on morphology and standard laboratory biochemical techniques alone, which can lead to misdiagnosis of human and animal *S. pseudintermedius* infections [7]. This review will focus primarily on *S. aureus* and S. *pseudintermedius*, the toxin genes they carry, and how they relate to infection in humans, dogs, and horses.

Staphylococcal infections and the rise of methicillin resistance

Methicillin resistance in staphylococci

Methicillin-resistant S. *aureus* (MRSA) and *S. pseudintermedius* (MRSP) infections are common in humans and animals [8,9]. Methicillin was the first penicillinase-resistant β-lactam antibiotic designed to combat staphylococcal infections and worked by inhibiting cross-linkage between peptidoglycans in the bacterial cell wall [10]. Methicillin is no longer used in clinical practice due to widespread resistance that was first noted in 1961, a year after its introduction [10]. Resistance to β-lactam antibiotics can be horizontally transferred between staphylococci through acquisition of the *blaZ* gene encoding a β-lactamase that imparts resistance to penicillin or the *mecA* cassette, which encodes an altered penicillin binding protein (penicillin binding protein 2a) that has a low affinity for all β-lactam antibiotics and imparts resistance to methicillin [11]. Methicillin was eventually supplanted by more effective and less toxic antimicrobials, but *mecA*-mediated β-lactam resistance is still classified as “methicillin”-resistance as an indicator for resistance to all β-lactam drugs. The *mecA* gene is carried on a horizontally

transferrable, MGE called the staphylococcal cassette chromosome *mec* (SCC*mec*) and can be transferred between coagulase-negative and -positive species of staphylococci [12].

Human infections

Staphylococcus aureus is a major cause of soft tissue infections and bacteremia in people, and is the leading cause of infective endocarditis [13]. An estimated 72,444 people in the US developed MRSA invasive infections in 2014 [3]. Invasive infections usually occur in tissues that are normally sterile, such as blood, bone, or urine [3]; and the rates of staphylococcal bacteremia have increased during the late 20th century. For instance, the yearly incidence of MRSA bacteremia in Canada increased from 0 cases per 100,000 people in 1991 to 7.4 per 100,000 in 2005 [14]. Clinical MRSA infections result in 11,285 deaths annually and cost $478 million to $2.2 billion in direct health care costs [3]. *Staphylococcus aureus*-associated cellulitis most commonly involves the feet and legs, but may also involve the hands, arms, torso, and face, and the histopathologic hallmark is subcutaneous abscessation [8]. *Staphylococcus aureus* causes a contagious localized dermatitis (bullous impetigo) in young children and a fatal, generalized blistering skin condition (staphylococcal scalded skin syndrome; SSSS) in infants [15,16]. Severe *S. aureus* skin infections can progress to necrotizing fasciitis in patients with a history of injectable drug use or secondary, immunomodulating clinical conditions, such as diabetes mellitus or hepatitis C [8].

Staphylococcus pseudintermedius is most frequently associated with skin/soft tissue infections and animal bite wounds in people, with rarer cases of bacteremia reported

[6,17,18]. *Staphylococcus pseudintermedius* can also colonize the nasal passages of people for prolonged periods, with veterinarians having higher colonization prevalence with MRSP than the general population [19,20], but colonization is often transient [21]. Once thought to be uncommon except in cases of dog bite, the prevalence of *S. pseudintermedius* infections in humans is difficult to determine due to frequent misidentification of *S. pseudintermedius* as *S. aureus* by commercial bacterial identification systems designed for humans [6]. *Staphylococcus pseudintermedius* infections in humans may often be misdiagnosed as *S. aureus* due to similarities in morphology and biochemical characteristics between the species, with one report describing a 13% (13/101) misdiagnosis rate [18]. Misdiagnoses of *S. pseudintermedius* infections in people are serious because there are differences in antimicrobial drug breakpoints between *S. pseudintermedius* and *S. aureus* which can lead to treatment failure due to MRSP being misidentified as methicillin-sensitive *S. aureus* [22].

Canine infections

While *S. aureus* infections do occur in dogs, *S. pseudintermedius* is the most common cause of pyoderma in dogs and is an important cause of post-surgical infections [23]. *Staphylococcus pseudintermedius* is also frequently cultured from the nares, pharynx, and perineal skin of asymptomatic, dermatologically healthy dogs [19,20]. *Staphylococcus pseudintermedius* can also carry SCC*mec* and can potentially transfer methicillin resistance to other *S. pseudintermedius* or *S. aureus* isolates [24]. The prevalence of methicillin-resistant *Staphylococcus* spp. colonization in healthy dogs varies

widely amongst regions, ranging from 6.5 - 73% [25,26], and the prevalence of MRSP and MRSA in healthy canine patients from referral hospital settings is reported to be between 0 - 6.2% and 0.5 - 1%, respectively [26-28]. Skin lesions attributable to S. *pseudintermedius* are often found on the ventral aspects of the abdomen and trunk, groin, muzzle, interdigital regions, and axilla [28]. Pyoderma usually presents in two forms, either as erythema of the skin with crusting papules or vesicles (superficial) or as alopecic nodules with extensive inflammation, draining tracts and surface ulceration (deep) [29]. As in humans, many dogs also have secondary clinical conditions that may predispose them to staphylococcal infections, such as autoimmune conditions, endocrinopathies, ectoparasitism, and paraneoplastic syndromes [30].

Equine infections

The prevalence of *S. aureus* colonization in horses that live on farms ranges from 1 to 8% in Canada [31] and between 4 and 39% in Europe [5,32]; and colonization prevalence rate in hospitalized horses was reported as 40-50% [32,33]. The nares of horses and other equids, as well as the carpal skin in hospitalized horses [33], are frequently colonized by methicillin sensitive *S. aureus* (MSSA). The prevalence of MRSA in healthy horses ranges from 0 to 6% [31,34], with most studies reporting a 4-5% prevalence [12,35-40]. While many horses colonized with *S. aureus* are asymptomatic carriers, MSSA and MRSA infections have been associated with significant morbidity and mortality in horses [39], and MRSA can be transferred between humans and horses and amongst groups of horses [41,42] resulting in important public health implications. Soft tissue, skin, surgical

incision, and joint infections are the most common manifestations of MRSA infection in hospitalized horses [43]. In one survey of bacterial infections in horses from Canada, *S. aureus* was more frequently cultured from cases with severe, chronic pleuropneumonia than other sites in the respiratory tract, such as the nares or guttural pouches, and *S. aureus* was more often cultured from post-surgical infections than traumatic wounds [44]. Risk factors for horses developing MRSA infections in veterinary hospital or community settings include administration of ceftiofur, aminoglycosides or other antimicrobials within the prior 30 days, and previous MRSA colonization of the patient or horses housed nearby [12,45].

Staphylococcus spp. strain typing schemes and their importance in epidemiology

S. aureus strains can be grouped into healthcare-associated (HA-MRSA) and community-acquired (CA-MRSA) based on the population in which the strain was first identified [46], but this designation can change over time. Genetic lineages of *S. aureus* and *S. pseudintermedius* can be grouped and categorized using several different typing schemes. MRSA and MRSP strains are grouped based on presence of specific components within the SCC*mec* cassette (Types I –XI) and differences in the direct repeat unit (*dru*) of the SCC*mec* cassette [47-49]. Both MS and MR *S. aureus* and *S. pseudintermedius* isolates can be typed based on similarities in the tandem repeat area of the staphylococcal protein A (*spa*) gene or within seven conserved genes in a technique called multi locus sequence typing (MLST) [50,51]. Interrelated *S. aureus* strains derived from a common ancestor can be further grouped into clonal complexes (CC), and some genetic lineages of

S. pseudintermedius have been grouped into CC [9,52]. An additional staphylococcal typing technique based on comparison of banding patterns generated via restriction enzyme digestion, pulsed-field gel electrophoresis (PFGE), was used to categorize strains before the development of the sequence-based methods mentioned above. Major strains were designated with the country of isolation and a number (i.e. USA 300) [53]. While PFGE is sensitive in determining similar and divergent strains, the technique is difficult to perform on large culture collections and it is harder to compare the results from different laboratories without obtaining reference strains [51], resulting in it gradually being supplanted by sequence-based typing methods for analysis of *S. aureus* strains.

SCC*mec* typing

The *SCCmec* is a MGE that can be transferred between staphylococci of the same or differing species [54]. The SCC*mec* integrates into the genome at a specific insertion sequence (ISS) within a 23S rRNA methyltransferase called *orfX*. As of 2017, there are eleven SCC*mec* types curated by the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) [55]. The basic components of SCC*mec* types I – X are the insertion sequence (IS431), *mecA* encoding the alternate penicillin binding protein 2a, a complete or truncated version of the *mecA* regulatory gene (*mecR1*), and cassette recombinase complexes (*ccr*, of which there are three genes and four allotypes) that are flanked by direct repeat sequences containing the ISS [54]. Some of the types also carry transposons (such as Tn4001, Tn554, or ψTn554) or resistance determinants to heavy metals, such as arsenic, cadmium, and copper. The

presence or absence of these genes or differences in component type/ arrangement determines the SCC*mec* type, and a series of multiplex conventional PCRs were developed to detect types I – VI [47]. Type XI SCC*mec* encodes the divergent *mec* gene, *mecC* (also known as the *mecA*$_{LGA251}$ homologue), that was recently discovered in bovine MRSA strains [56]. One study in the early 2000s found that CA-MRSA strains from the central US were more likely to carry SCC*mec* type IV when compared to HA-MRSA [46].

S. pseudintermedius MR isolates carry type II, IV, or V SCC*mec* but can also carry a hybrid type II-III SCC*mec* that contains elements of *Staphylococcus epidermidis* SCC*mec* type II and *S. aureus* SCC*mec* type III [57]. SCC*mec* type VII, also called VII-241, was discovered in a MRSP isolate from Switzerland [57]. Strains of MRSP prevalent in Israel carry a pseudo-SCC*mec*, ψSCCmec$_{57395}$, which carries *mecA* and heavy metal resistance determinants but lacks *ccr* genes [58]; one MRSP isolate cultured from a dog in Thailand carried a novel SCC*mec* composite island called SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI that encodes restriction modification and heavy metal resistance genes [59].

***Spa* and *dru* typing**

Two single gene typing schemes currently in use for staphylococci are based on identifying variable-number tandem repeat (VNTR) sequences in specific regions (X-regions) of the staphylococcal protein A (*spa*) gene and SCC*mec* direct repeat unit (*dru*) region. The *spa* typing schemes can be used for both MR and MS *S. aureus* and *S. pseudintermedius*, and *dru* typing is used for MR staphylococci [48-50,60,61]. The *spa* gene encodes an IgG binding protein and is carried as a single copy in *S. aureus* but can

occur as two orthologues carried in succession in *S. pseudintermedius* (also designated as *spsP* and *spsQ*) [62], with *spsQ* containing the X-region that determines *spa* type in this species. The PCR primers originally developed to amplify *spa* [50] in *S. pseudintermedius* often do not bind specifically to the correct *spa* gene, resulting in a high proportion of isolates that could not be *spa* typed due to non-specific amplification of products; this anomaly was corrected with primer redesign in a later study which increased the number of *spa*-typable isolates [9]. The *spa* typing naming schemes for *S. aureus* and *S. pseudintermedius* are not interchangeable and are curated by different groups (*S. aureus*- Ridom SpaServer, http://spa.ridom.de/spatypes.shtml; *S. pseudintermedius*- Arshnee Moodley). *Dru* typing is based on a similar method of identifying 40 bp-long VNTR sequences in the direct-repeat unit region of SCC*mec* adjacent to IS431 in *S. aureus* and *S. pseudintermedius* [48,61]. The online *dru* typing database (http://dru-typing.org/site/) is currently curated by Richard V. Goering. As of October 2017, there are 17,278 *spa* types for *S. aureus*, 81 *spa* types for *S. pseudintermedius*, and 530 *dru* types for MRSA and MRSP.

Multi locus sequence typing (MLST) and ribosomal MLST (rMLST)

The multi locus sequence typing (MLST) method is based on determining allelic variation in highly conserved genes with specific combinations of gene allele types determining overall sequence type (ST). The *S. aureus* MLST is based on allelic variation in seven conserved genes: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glp*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*),

triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*) [51,63]. Founding STs of CC are determined by identifying STs that have the greatest number of single-locus variants (i.e. isolates that differed from the most STs at only a single locus). The major CC associated with human disease worldwide are USA100 (CC5), USA200 (CC30), USA300 (CC8), USA400 (CC1), and USA600 (CC45) [53]. In the US, CC5 strains are associated with nasal colonization and invasive infections [64], while CC30 is associated with mucosal infections and toxic shock syndrome in women [65]; CC1 is the predominant group in Canada and most isolates are *spa* type t127 [66]. Most epidemic CA-MRSA infections in the US are caused by CC8, and these strains are often carry multiple cytolytic toxin genes [53,67]. In European horses, the most prevalent MRSA strains are ST398, 8, and 612 [34]. In Canada, ST8-SCC*mec* type IV MRSA strains have also been described in veterinary hospital-associated outbreaks [43]. While the first reports of MRSA in horses came from the US [68] in the 1990s and a few subsequent reports [69,70] have characterized MRSA from horses in the US utilizing current sequence-based typing methods, none of the US or Canadian studies have included MSSA derived from horses with infections.

Since the 2010s, MLST schemes have been developed for *S. pseudintermedius* that have allowed more precise characterization of isolates into ST and grouping of a few genetic lineages into CC [52,71]. The *S. pseudintermedius* MLST is based on allelic variation in seven conserved genes: elongation factor Tu (*tuf*), 60 kDa chaperonin (*cpn60*), phosphate acetyltransferase (*pta*), adenylosuccinate synthetase (*purA*), formate dehydrogenase (*fdh*), sodium sulfate symporter (*sar*), and acetate kinase (*ack*) [71]. To

date, only a few *S. pseudintermedius* isolates derived from human infections have been characterized by MLST [18,71-73]. The most common ST in a recent survey that included canine-derived isolates collected from all geographical regions of the US were 68, 71, and 84, with each of these ST being founders of CC that encompassed dozens of other ST [52]. Most of the MRSP isolated are descended from a few clones, while methicillin-sensitive *S. pseudintermedius* (MSSP) isolates do not spread clonally and have diverse ST [18,52]. ST71 is frequently associated with SCC*mec* type II-III [57] and is a unique ST because it includes both MRSP and MSSP lineages, whereas other groups, such as ST68 and 84, are composed of MRSP [52]; this finding suggests that there may be ready transfer of antibiotic resistance genes between MRSP and MSSP members of this lineage. In one case, a human-derived MRSP ST71 strain also demonstrated a reversible small colony variant phenotype similar to *S. aureus* that imparted resistance to multiple antimicrobials [72]. ST71 and ST68 MRSP have also been frequently associated with epidemic outbreaks in veterinary hospitals in Europe and the US, respectively [74]. Methicillin-resistant ST71 strains have been associated with nasal infection and colonization of immunocompromised patients [72,73] with and without ownership of pets. The ST71 MRSP lineage is also the only type documented to be associated with an outbreak of blistering skin lesions in diabetic human hospital patients, the majority without demonstrated contact with dogs [75].

For determining ribosomal MLST (rMLST) in staphylococci, the allelic variation in 51 non-paralogous ribosomal genes is compared to assign type. The 53-gene rMLST scheme can be used to type any genus/species of bacteria, but as most staphylococci have

two copies of the *rpsN* and *rpmG* genes, these genes are excluded from the typing scheme for staphylococci [76,77]. Differences in the alleles for any one of the genes constitutes a new rMLST. Of the typing methods discussed in this review, rMLST is the newest method and the one with the least published data because the technique is not PCR based and was developed for isolates that have been subjected to whole genome sequencing (WGS). As of October 2017, there are over 4,500 rMLST of *S. aureus* deposited in the rMLST database on the online Bacterial Isolate Genome Sequence Database (BIGSdb) genomics platform (https://pubmlst.org/rmlst/; curator- Keith Jolley) [78]. In contrast, there are only 23 rMLST reported for *S. pseudintermedius* in the database. Some of the most common rMLST for livestock-associated *S. aureus* strains from Europe based on querying the BIGSdb are: 4290 (ST1), 4340 (ST398), and 4499 (ST133); while common MRSA rMLST are 4326 (ST5) and 4320 (ST8). The predominant *S. pseudintermedius* rMLST are 17490 (ST71), 17491 (ST68), and 48490 (ST84) based on BIGSdb data.

Classes of staphylococcal toxins

Staphylococcal toxins are divided into three general classes: exfoliative, membrane damaging toxins, and superantigens (SAgs). Carriage of specific toxin genes is prevalent within certain genetic lineages of S. *aureus*, and most isolates carry multiple SAgs and other toxin genes. Toxin genes can be horizontally transferred between staphylococci and are often carried on large MGE called pathogenicity islands (PI), bacteriophages that integrate into the bacterial genome as prophages, or plasmids [79].

Exfoliative toxins

Exfoliative toxins (ET) are potent serine proteases that are secreted by staphylococci and are thought to facilitate infection through cleavage of the keratinocyte junctional protein desmoglein-1 (Dsg1) in the stratum granulosum, resulting in extensive blistering and separation of the superficial layers of the epidermis and sparing of the basal layers [80]. In *S. aureus*, the four recognized ET are ETA, ETB, ETC, and ETD, with ETA and ETB being the most associated with disease [81-83]. *Staphylococcus aureus* strains responsible for bullous impetigo and SSSS in children and infants often carry ETA and ETB [15,16]. Carriage of e*ta* and *etb* has also been documented in *S. aureus* strains isolated from wounds, implant, and urinary tract infections [84-87], with the *eta* gene being more prevalent in isolates from North America and Europe and *etb* being more prevalent in isolates from Japan [83]. ETC has only been described in one isolate that was collected in the 1990s from a horse with dermatitis [81]. However, the DNA sequence encoding ETC and other structural properties of the protein have not been reported, and the ETC carrying reference strain (Horse-1) has never been sequenced or banked in a commercial repository for study. ETD was identified in 2002 in *S. aureus* N315 and is encoded on a 9,054 bp PI [82]. ETD has been isolated from samples collected from adults with furunculosis and skin abscesses, and while it does cleave Dsg1 experimentally, its overall significance in *S. aureus* pathogenesis is unknown [82,86]. In young pigs, SHETA and SHETB expression in *S. hyicus* is associated with exudative epidermitis, which results in epidermal blistering similar to SSSS [83]. The four proposed ET genes in *S. pseudintermedius* are *speta*, *siet*, *expA* (formerly known as *exi*), and *expB* [88,89]. The

potential for horizontal transfer of the *S. pseudintermedius* ET genes has not been investigated, and it is unknown if the genes are carried on MGE. While studies with recombinant EXPA and EXPB have demonstrated that they target canine Dsg1, the protein crystal structures of the *S. pseudintermedius* ET have not been evaluated.

Membrane damaging toxins

Pore-forming toxins

The main function of pore-forming toxins is to damage the cellular membrane of the host cell, which can cause cellular lysis and necrosis of tissues. Pore-forming toxins produced by staphylococci include: α-hemolysin (Hla), leukocidins, and phenol-soluble modulins [90]. Approximately 95% of all *S. aureus* produce Hla, which is responsible for lysis of a wide variety of cell types, including erythrocytes, mononuclear leukocytes, and epithelial cells [90]. Hla was one of the first identified *S. aureus* toxins and causes hemolysis of erythrocytes through binding of surface a disintegrin and metalloprotease 10 (ADAM10) receptors. While highly cytolytic to rabbit erythrocytes, Hla does not cause hemolysis of human erythrocytes due to ADAM10 expression differences on the surface of erythrocytes between the two species [44].

The leukocidins have been associated with recurrent soft tissue infections and necrotizing pneumonia in people [90]. Leucocidins contribute to immune system evasion through lysis of neutrophils, monocytes and macrophages. Leucocidins are composed of two class S and F protein subunits that oligomerize to form a β-barreled pore, and the degree of cellular lysis induced by each leucocidin differs amongst host species [90,91].

The leucocidins of importance to human and livestock infections are γ-hemolysin, leucocidin AB (LukAB), leucocidin ED (LukED), Panton-Valentine leucocidin (LukSF-PVL), leucocidin PQ (LukPQ), and leucocidin MF' (LukMF'). Gamma-hemolysin is formed by interaction of the HlgAB and HlgCB components, which bind to phagocyte chemokine (CXCR1, CXCR2 and CCR2) and complement (C5aR and C5L2) receptors, respectively, for targeted killing of monocytes and neutrophils [92]. LukAB (also known as LukGH) kills human neutrophils by binding to the Mac1 integrin and is carried by most isolates [93,94]. LukED has broad targeting action with different chemokine receptors and can kill neutrophils, T-lymphocytes, macrophages, and dendritic cells, effectively disarming both the host's innate and adaptive immune responses [95]. LukED and γ-hemolysin carriage are ubiquitous in *S. aureus*, with approximately 90-100% of isolates carrying both genes [16,96].

LukSF-PVL is highly toxic to human and rabbit neutrophils, and PVL carrying strains have been associated with necrotizing pneumonia in murine models of human infection [97]. LukPQ has been implicated as a cause of pneumonia, and LukMF' is often secreted in bovine mastitis cases [91,98]. The newly categorized LukPQ and LukMF' specifically target equine and ruminant neutrophils, respectively [91,98]. LukPQ-positive isolates have only been described in isolates collected from horses in Europe, and LukPQ was not present in a small collection of *S. aureus* isolates collected from horses in Ohio [91]. Leucocidins are encoded on bacteriophages that integrate into the bacterial host's genome (prophages) with PVL encoded on Φ-PVL, LukPQ on ΦSaeq1 (deposited as the strain 3711 prophage), and LukMF' on Φ Sa1 [91]. A similar two component leucocidin,

LukS-I and LukF-I, has been identified in *S. intermedius* isolates from pigeons and *S. pseudintermedius* isolates from dogs, but the significance is unknown [99].

Phenol-soluble modulins (also known as δ- hemolysins) are peptides with cytolytic and pro-inflammatory properties that are ubiquitously carried in *S. aureus* and may have a role in initial colonization of the host through formation of biofilms [90]. BacSp222 is a peptide produced by *S. pseudintermedius* that lyses other Gram-positive bacteria and has characteristics of a bacteriocin [100].

Beta-hemolysin

Beta-hemolysin (*hlb*) does not form pores in the cell membrane and is a sphingomyelinase that hydrolyzes the lipid sphingomyelin in the plasma membrane of host cells, which results in a conversion of sphingomyelin into ceramide and phosphorylcholine. The specific action of β-hemolysin that causes cell death is not known, but it is thought to alter cell membrane fluidity and disrupt cellular signaling by causing cholesterol to aggregate in the lipid bilayer [101]. Beta-hemolysin carriage is common, with approximately 70% of equid *S. aureus* isolates carrying *hlb* in one report [96].

Superantigens

The staphylococcal SAgs cause mass, indiscriminate stimulation of T-lymphocytes through bridging of the variable β-chains with α or other β-chains of major histocompatibility complex (MHC) class II molecules without the need for antigen presentation. The resulting cytokine release by T-lymphocytes and other antigen

presenting cells can cause potential shock and organ failure in the host. Most *S. aureus* strains carry 5-6 SAgs genes [90]. The *S. aureus* SAgs include toxic shock syndrome toxin (TSST-1 or SEF), enterotoxins A-E, G-J, and R-T, and the enterotoxin-like (SEl) agents K-Q, U, V, and X [90]. Secretion of TSST-1 into the blood induces fever and blood pressure changes within the host, and TSST-1 was first associated with shock in menstruating women with a history of usage of high absorbency tampons [102]. In animals, TSST-1 carriage has frequently been reported in isolates cultured from goats, and TSST-1 expression has been associated with a toxic shock-like syndrome in a horse with pneumonia [102,103]. Enterotoxins are associated with staphylococcal food poisoning and induce vomiting in primates [90]. Carriage of SEA and SEC is common in USA200 and USA400 strains, while carriage of enterotoxins in the PVL-positive USA300 strains is uncommon [53]. Enterotoxin H carriage is predominately associated with CC1 lineage, while *ser* and *sej* carriage is associated with CC5 MRSA [104]. Enterotoxins D and R are often carried separately or together on plasmids [105].

Experimentally, the SEl toxins have similar properties to enterotoxins, but have not been proven to induce vomiting in humans or primate models [90]. Most of the SEl proteins have not been extensively studied, but expression of SEl-X is associated with necrotizing pneumonia in people with MRSA and development of diabetic foot ulcers [106,107]. The enterotoxin genes *seg* and *sei*, and SEl genes *sel-m*, *sel-n* and *sel-o* are encoded on a PI in a grouping called the enterotoxin gene cluster (*egc1*) [108] or if containing *sel-u* or variant *sel-u, egc2* or *egc3*, respectively. The *egc* is commonly encountered in CC5 or CC30 MRSA [104]. Most enterotoxins and SEl are encoded on

MGE carried by bacteriophages, PIs, or plasmids, and carriage is variable among isolates, with an exception being SEl-X which is encoded on the core genome and occurs in approximately 90% of *S. aureus* isolates [106,107]. Carriage of SEl-K, SEl-Q, and SEl-X is commonly encountered in USA300 strains [53]. Carriage of SEl toxins has not been evaluated in *S. aureus* isolates from horses. In *S. pseudintermedius*, the sec_{canine} and *se-int* genes have been identified as possible enterotoxins, but their importance in clinical disease is unknown [109,110]. Analogs to the *S. aureus* TSST-1, SEA, SEB, SEC, and SED enterotoxins have been detected via PCR in *S. pseudintermedius* isolates from dogs with and without pyoderma [111,112], but whether the PCR amplicons represented the targeted *S. aureus* SAg genes has not been determined as DNA sequencing of the amplicons was not done to determine if the PCR primers were specific to the enterotoxin genes investigated.

Relationships between toxin gene carriage, strain type, and antibiotic resistance

The secretion of exoproteins, including toxins, in staphylococci is controlled by the accessory gene regulator (*agr*) locus. The *agrD* gene encodes a precursor for a peptide (autoinducing peptide; AIP), of which there are four different types, that is used in quorum sensing between staphylococci and is capable of cross inhibiting gene expression pathways in staphylococci producing a different type of AIP [80,113]. The *agr* type is closely linked to the genetic lineage of a strain, determined through the strain typing methods, and certain *agr* types have been associated with specific types of infection in people. The methods for determining *agr* type in *S. aureus* and *S. pseudintermedius* are

different and are not interchangeable [113,114]. Exfoliative toxin producing strains of S. *aureus* are often associated with type IV *agr* systems [80], while bullous impetigo is associated with type I and endocarditis is more associated with types I and II [113]. While a definitive link between type of infection and *agr* system has not been demonstrated in *S. pseudintermedius*, *agr* type III is commonly observed in the predominant European MRSP strain ST71 [115].

Methicillin resistance in *S. aureus* and S. *pseudintermedius* spreads clonally, with a few predominant, interrelated strains spreading through a human or animal population or geographic area due to antimicrobial selection pressures, with MRSA strains often concurrently carrying leucocidin genes, ET, or other virulence factors [9,16]. Carriage of toxin genes often coincides with antimicrobial resistance, carriage of other virulence genes, or strain type in *S. aureus*. For instance, the predominant MRSA strain in North America is USA 300, which is a PVL carrying SCC*mec* type IV strain that rose to prominence in 2011 [116]. Methicillin-resistant *S. aureus* ST8 and SCC*mec* type IV isolates frequently carry a virulence element, the arginine catabolic mobile element (ACME), which enhances the ability of carrying strains to colonize human skin [117], but this element is not prevalent in livestock associated strains of MRSA [118]. The *S. aureus* phenol soluble modulin gene, *psm*, is located predominately on SCC*mec* II, III, and VIII, and is transferred at the same time as methicillin resistance genes between isolates [119]. In European horses, one of the most prevalent *S. aureus* strains, ST398 [120], frequently carries the prophage that encodes *lukPQ* [91]. Carriage of *lukPQ* is also associated with other CC typically associated with livestock, such as CC1, CC133, and CC1660 [91]. The

plasmid that encodes *etb* in *S. aureus* also carries a cadmium resistance operon and an epidermal cell differentiation inhibitor (EDIN) called ADP-ribosyltransferase (*edinC*), a Rho GTPase-inactivating protein, which interferes with actin cytoskeleton regulation in bacteria. Strains that carry the *etb* plasmid are also more likely to carry *edinB* on the chromosome, a gene that is involved in the development of bacteremia in pneumonia patients with community acquired MRSA [121,122]. *Staphylococcus aureus* strains that carry *etb* are more likely to be methicillin sensitive [123], whereas *eta* carrying isolates often have strong clonal associations and are frequently methicillin-resistant [15,16]. While *S. aureus* virulence factors have been studied for many years, it is unknown if carriage of toxin genes or other virulence factors coincides with particular strains or genetic lineages in *S. pseudintermedius*.

CHAPTER III

S. PSEUDINTERMEDIUS EXFOLIATIVE TOXIN GENE CARRIAGE IN ISOLATES FROM DOGS (2007–2013)

Introduction

The four proposed ET genes in *S. pseudintermedius* are *speta*, *siet*, *expA* (formerly known as *exi*), and *expB* [88,89,124,125]. Previous studies that have included isolates from healthy and diseased dogs have reported prevalence rates of 0 - 23% for *expA* and 2 - 28% for *expB*, but the sample sizes were relatively small [112,115,126-130]. In contrast, carriage of the *speta* and *siet* genes has been highly prevalent in previous studies, with almost universal carriage in the examined isolates [115,131]. The purpose of this study was to determine the prevalence of ET gene carriage in *S. pseudintermedius* isolates cultured from dogs with pyoderma, other clinical conditions, and dermatologically healthy dogs in a referral hospital population. We hypothesized that there would be differences in ET gene carriage between healthy and diseased dogs and that ET gene carriage would differ based on patient demographics, type of infection, methicillin-resistance status and/or genetic lineage of the isolate.

Using a subset of the dogs in the prevalence study that also had skin biopsies collected within the same time frame as the bacterial culture, we evaluated the severity, type, and depth of inflammation in hematoxylin and eosin (H&E) stained, FFPE skin biopsies. The purpose of this analysis was to evaluate if there was a difference in inflammation severity and/or type of inflammation between dogs in the prevalence study.

We hypothesized that there would be differences between inflammation severity, type of inflammation, and depth of inflammation in skin biopsies collected from dogs that cultured *S. pseudintermedius* isolates of differing ET carriage profiles; we expected that the *speta+siet+expA* and the *speta+siet+expB* profiles would be associated with more severe inflammation in the paired biopsies.

Materials and methods

Strain collection and demographics

All *S. pseudintermedius* isolates used in this study were part of an institutional veterinary clinical strain collection composed of 500 isolates collected from dogs that presented to the Texas A&M University Veterinary Medical Teaching Hospital from 2007-2013. The 500 isolates were collected from 475 individual dogs. Within this population, there were 359 isolates (72%) that were residual patient diagnostic samples collected from dogs with various clinical conditions, of which pyoderma (n = 99), surgical site infections (n = 38), and urinary tract infections (n = 59) represented the most frequent culture sites. The database also included 141 isolates (28%) collected as part of a staphylococcal screening study that cultured the nares and perineal skin of dermatologically healthy dogs that presented for orthopedic surgery evaluation. Sampling conformed to the ethical guidelines and standards of care for the hospital, and the animal use protocol for the collection of samples from the healthy dog group was approved by the institutional animal care and use committee (AUP 2010-068).

The mean age of dogs from which isolates were derived was 6.3 years (SD = 3.65 years; median = 6 years; range, 2 months to 16 years). There were 238 isolates from spayed females, 24 isolates from intact females, 181 isolates from castrated males, and 57 isolates from intact males. Eighty-eight breeds were represented and there were 57 mixed-breed dogs. The majority of the isolates came from dogs that were classified into the AKC Sporting (n = 127; 25%) and Toy (n = 70; 14%) groups. Labrador retriever (n = 76; 16%) was the most frequent breed encountered in the diseased and healthy groups.

Isolates were identified as *S. pseudintermedius* at the time of initial diagnosis or screening using a combination of standard laboratory techniques, such as evaluation for coagulase and urease production, the ability to grow in the presence of 6.5% sodium chloride, and the ability to ferment trehalose and mannitol, and confirmation with a diagnostic staphylococcal speciation PCR using previously described methods [1,132]. Antimicrobial resistance was determined at the time of initial culture through minimum inhibitory concentration testing, and methicillin resistance was determined via *mecA* PCR, and oxacillin and cefoxitin disk diffusion as previously described [22]. Isolates were preserved in lysogeny broth with 20% glycerol and frozen at -80ºC until DNA extraction was performed.

DNA extraction and molecular techniques

Isolates were struck onto Mueller-Hinton agar with a 96-pin replicator and incubated at 37°C overnight. A single colony was inoculated into 1 mL of lysogeny broth in 96-well format and incubated at 37°C overnight for DNA extraction. Genomic DNA

was extracted from the samples using a Wizard® SV 96 Genomic DNA Purification System (Promega Corporation, Madison, WI, USA) as per the manufacturer's instructions, except an enzymatic lysis buffer composed of 50mM EDTA and 40 mg/mL lysozyme was used in place of the included kit lysis buffer and samples were incubated at 37°C for 2 h with lysis buffer before vacuum extraction. The PCR primers used for each of the ET genes are listed in **Table 1**.

Gene	**Forward primer (5' → 3')**	**Reverse primer (5' → 3')**	**Amplicon size (bp)**	**Reference**
speta	AAAAAGCGCCTTTAGCAACA	GTGAACACAGATGCGACCAG	201	This study
siet	GGATCCACAAGTACTGTACTTGAAGAAGG	AAGCTTCCCTTCATATTTGTACATTGGTCGT	875	[88]
expA (exi)	TAAGCATGCAATCATATAATGAGGAAGAAATATTAAAAAAGCAA	TCTGGATCCTTCTTCTTGTAATTTAGCTCTTTTTTTCAAGTCTTC	755	[88]
expB	CGCCTGGCGTATATGCTAAAAC	TTCAGCGCCAAATTGTCCAT	326	This study

Table 1: PCR primers for amplification of ET genes. The underlined regions indicate insertion of SphI (GCATGC), BamHI (GGATCC) and HindIII (AAGCTT) restriction enzyme cut site sequences in the original *siet* and *expA* primers [88].

PCR reactions consisted of 1 x Buffer (20 mM Mg2), 2.5 mM each DNTP, 0.2 µM each primer, 1.25 U of Takara Ex Taq™ (Takara Bio, Inc., Kyoto, Japan), and 25-100 ng template per 50 µl sample. PCR thermocycler conditions for the ET genes consisted of an initial denaturing step at 94°C for 5 min; 30 cycles of denaturation at 94°C for 1 min,

annealing at either 55°C (*expA*, *expB*, or *speta*) or 60°C (*siet*) for 1 min, and extension at 72°C for 1 min; and a final elongation step of 72°C for 7 min. SCC*mec* typing via multiplex PCR for *ccr* type I-VI and class A-C *mec* assignment was performed on all MR isolates as previously described [47]. PCR screening for the hybrid *Staphylococcus epidermidis* Type II and *S. aureus* Type III SCC*mec* elements (Type II-III) and for SCC*mec* Type VII-241 were also conducted as previously described [57]. All of the isolates from pyoderma and surgical site infections, all of the MR isolates in the healthy group, and a random selection of MS and MR isolates (n = 82) cultured from other body sites in the diseased group were *spa* typed using the original [50] and modified [9] primer sets and *dru* typed [48] as previously described. Amplicons were visualized via electrophoresis at 110 V on a 1% agarose gel stained with GelRed™ (Biotium, USA).

Selected PCR reactions were purified with a NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) as per the manufacturer's instructions. As there were not any ET-specific *S. pseudintermedius* reference strains at the time of writing this manuscript, the specificity of each ET primer set was validated through sequencing. Respective amplicons targeting the *speta*, *siet*, *expA*, and *expB* genes, and the *spa* and *dru* repeat regions of select isolates were submitted for Sanger sequencing (Eton Biosciences, Inc., San Diego, CA, USA). The ET gene nucleotide sequences were queried against the NCBI reference database using the nucleotide Basic Local Alignment Search Tool (BLAST). The *speta*, *siet*, *expA*, and *expB* amplicons had 99% identity to their respective reference sequences in GenBank: NC_014925.1 (formerly ADX77621.1), AB099710.1, AB489850.1, and AB569087.1. *Spa* and *dru* types were assigned as described previously

based on tandem repeat comparison to the respective databases (*spa* curator- Dr. Arshnee Moodley and *dru* database- www.drutyping.org).

Slide scoring systems

The H&E stained, FFPE skin biopsies were examined for 25 of the dogs evaluated in the ET prevalence study. The skin biopsies were residual diagnostic specimens submitted by the attending clinician during the same time period as the bacterial cultures (i.e. paired biopsies), but did not necessary represent the exact region cultured. The slides for each of the cases were blinded by an individual that had not seen the ET carriage prevalence data and were read by a board certified veterinary anatomic pathologist (LKB). A grading scale was developed to subjectively score the severity of inflammation. Briefly, < 10 inflammatory cells per field was minimal (score 0); > 10 and ≤ 50 was mild (1); > 50 and ≤ 300 was moderate (2); and > 300 was severe (3). Histologic representations of the severity score scale are given in **Fig. 1**. An individual score was given for each of ten 400x (40x objective and 10x ocular lens) fields within areas of inflammation on the slide, and the results from each field were averaged to produce an overall inflammation severity score for the slide. If more than one slide of a similar lesion was evaluated for a single case, the slide with the more severe inflammation score was used in the final analysis after un-blinding of the slides.

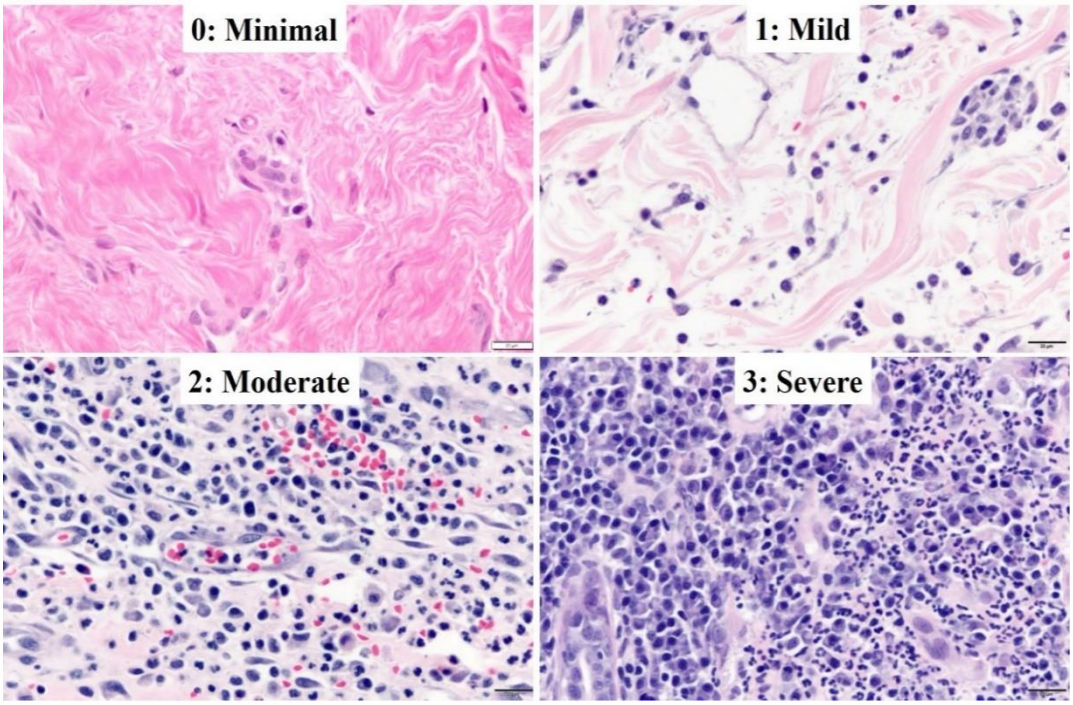

Figure 1: Inflammation scoring system examples. Representative 400x fields of each of the score categories in the H&E stained skin biopsies (Olympus DP74 microscope camera; Tokyo, Japan). The scale bars represent 20 µm.

Inflammation type was divided into polymorphonuclear (neutrophils and eosinophils), mononuclear (encompassing lymphocytic, plasmacytic, and histiocytic inflammatory cells), and a combined mononuclear/ polymorphonuclear group. Assignment of type was based on which group represented > 60% of the inflammatory cells within the H&E section. If sections contained equal numbers of polymorphonuclear and mononuclear (1:1) inflammation, the type was classified as mononuclear/ polymorphonuclear. The depth of inflammation was classified as superficial if the majority was localized to the epidermis or superficial dermis or classified as deep if inflammation extended into the deep dermis or underlying panniculus, muscle or

cartilaginous structures. The pathologist also noted if coccoid bacteria were visible in the H&E section.

Statistical analyses

All analyses for the prevalence study were performed using S-PLUS statistical software (Version 8.2, TIBCO, Seattle, WA). To assess for relationships between breed and ET gene carriage, the purebred dogs were categorized based on American Kennel Club Group (i.e. Sporting, Hound, Toy, etc.) [133]. For continuous variables (e.g., age), linear mixed-effects modeling was used when possible to account for some isolates being from the same dog. For comparisons of toxin type (i.e. presence or absence of toxin gene carriage), random-effects logistic regression was used to account for the correlation of some dogs contributing multiple isolates. When there were 0 values in some cells, random-effects logistic regression was not possible and organisms were considered without regard for correlation among observations from the same dog. These categorical variables were compared using chi-squared or Fisher's exact test when expected cells had counts fewer than 5. A P value less than 0.05 was considered significant. All statistical analyses for the inflammation scoring study were done with JMP® Pro 12.2.0 (SAS Institute, Inc., Cary, NC). Categorical variables were also compared using Fisher's exact test when expected cells had counts fewer than 5 or a two-tailed Fisher's exact test. A P value less than 0.05 was considered significant in both studies.

Results and discussion

Prevalence study

ET gene carriage

Of the 500 isolates, 100% (500/500), 99% (497/500), 13% (67/500), and 16% (80/500) were positive for the *speta*, *siet*, *expA*, and *expB* ET genes, respectively. The gene combinations (**Fig. 2**) observed were: *speta* alone (n = 3), *speta+siet* (n = 357), *speta+siet+expA* (n = 60), *speta+siet+expB* (n = 73), *and speta+siet+expA+expB* (n = 7). *The expA* and *expB* genes were always carried concurrently with *siet*. Because of the ubiquitous carriage of *speta* and *siet*, the statistical analyses focused on carriage of *expA* or *expB*.

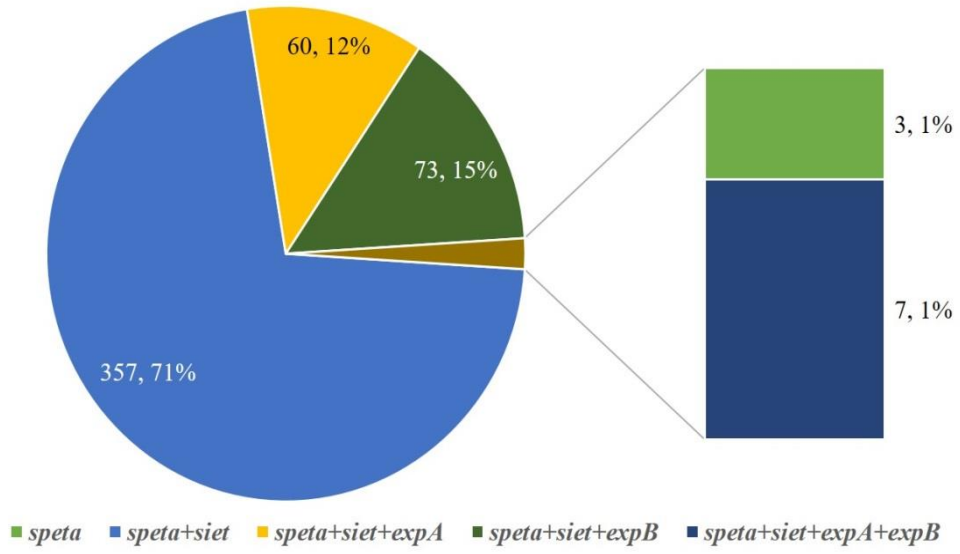

Figure 2: *S. pseudintermedius* ET carriage profiles observed in isolates from dogs.

There was no correlation between carriage of *expA* or *expB* by the isolate and sex of the associated dog (P = 0.34 and 0.064, respectively). The ages of dogs with isolates that were *expA*-positive or *expB*-positive did not differ (P = 0.9971 and P = 0.8767, respectively). The proportion of *expA and expB* positive isolates did not differ by American Kennel Club (AKC) group (P = 0.9875 and 0.5913, respectively). Although the proportion of *expA*-positive isolates was lower among those from diseased dogs (12%; 43/359) than healthy dogs (17%; 24/141), this difference was not significant (P = 0.1790). The proportion of isolates from diseased dogs that were positive for *expB* (16%; 56/359) was also similar to that of isolates from healthy dogs (17%; 24/141).

When considering both the healthy and diseased groups, the odds of pyoderma were 2.5 times greater for dogs that were *expA*-positive relative to dogs that were *expA*-negative (95% CI, > 1.0 to 6.3; P = 0.0439). The odds of pyoderma were not significantly different for *expB*-positive healthy and diseased dogs (P = 0.1741). The odds of surgical site infection were also not significantly different for *expA* or *expB*-positive healthy and diseased dogs (P= 0.0582 and 0.1741, respectively).

Antibiotic resistance

Resistance to ampicillin and doxycycline was frequently encountered in the isolates, occurring in 78% (388/497) and 40% (133/331) of the isolates, respectively. Resistance to erythromycin (33%; 162/496), trimethoprim sulfonamide combination drugs (30%; 151/496), enrofloxacin (27%; 136/497), and gentamicin (24%; 117/496) was also prevalent, while chloramphenicol (14%; 70/497) and rifampin (4%; 13/331) resistance

was low. There were 159 methicillin-resistant (MR) *S. pseudintermedius* isolates; 147 in the diseased dog group and 12 in the healthy dog group. All of the *speta+siet+expA+expB* and *speta* only isolates were methicillin-sensitive (MS). Of the 99 isolates from dogs with pyoderma, 49 isolates were MR and 50 were MS. The proportion of pyoderma isolates that were *expA*-positive was similar among those that were MR (18%; 9/49) and MS (20%; 10/50) (P = 0.9609). Using random-effects logistic regression to account for clustering among observations, the odds of dogs with MR isolates being *expA*-positive (1.2; 95% CI, 0.2 to 8.1) were not significantly (P = 0.8190) greater than for MS isolates. Of the 38 isolates from surgical site infections, 19 were MR and 19 were MS. As only one and three isolates were *expA* and *expB*-positive, respectively, odds ratios could not be computed.

Typing characteristics

SCC*mec* typing was performed on all MR isolates and yielded 65% (103/159) Type V, 16% (26/159) Type II-III, 7.5 % (12/159) Type IV, 1.8% (3/159) Type II, 0.6% (1/159) Type VII-241, and 8.8% (14/159) untypeable using the current conventional PCR typing schemes. Of the 144 isolates that could be SCC*mec* typed, 8% (11/144) were *expA*-positive and 9% (14/144) were *expB*-positive. *Spa* type was successfully determined for 52% (68/131) of the MR isolates tested. There were 22 distinct *spa* and 19 *dru* types. The most common *spa* types for the MR isolates were t06 (n = 43) and t02 (n = 11), and the most common *dru* types were dt11a (n = 29), dt11af (n = 21), and dt9a (n = 17). The most common SCC*mec*, *spa*, and *dru* type combinations were V+t06+dt11a (n = 20) and V+t06+dt11af (n = 11). Five new *spa* types were identified and added to the database: t77

(n = 2; r01-r02-r03-r03-r04-r05), t78 (n = 1; r01-r09-r02-r02-r03-r13-r13-r03-r06-r05), t79 (n = 1; r37-r03-r03-r06-r05), t80 (n = 1; r01-r21-r21-r21-r02-r03-r03-r06-r05), and t81 (n = 2; r01-r09-r02-r02-r03-r13-r03-r13-r03-r06-r05). Two new *dru* types were identified and added to the database: dt11bl (n = 5; 5a-3c-4a-0-3c-5b-3a-2g-3b-4e-3e) and dt13x (n = 1; 5a-2d-4a-1d-3a-5b-3a-2g-6o-3a-2g-3b-4e).

There was a strong association between SCC*mec* type and *dru* type (P < 0.0001) with dt9a over-represented for SCC*mec* type II-III, and dt11a and dt11af occurring with type V. The majority (7/8) of the type IV isolates that were sequenced were dt10h. *Spa* type and *dru* type were also strongly associated (P < 0.0001) with dt9a over-represented for t02, and dt11a and dt11af strongly associated with t06. There was no significant association with *expA* or *expB* carriage with the t06 and t02 *spa* types versus other types (P = 0.9782 and 0.1399, respectively). There was also no significant association with *expA* or *expB* carriage with the three most common *dru* types (0.1387 and 0.3106, respectively). S*pa* type was not correlated with pyoderma or surgical site infections among all isolates (P = 0.5499 and 0.9498, respectively). The proportion of isolates collected from dogs with pyoderma was significantly (P = 0.0159; chi-squared test) greater among dt11af isolates (62%; 16/26) than other isolates (33%; 27/83), and the proportion of isolates that were from dogs with surgical site infections was significantly (P = 0.0017; chi-squared test) greater for dt9a isolates (44%; 8/18) than for other *dru* types (11%; 10/91).

Conclusions

This is the first study with > 200 isolates that has looked at ET gene prevalence in S. *pseudintermedius* isolates from dogs. Carriage of *speta* was universal and *siet* was carried by 99% of the isolates. These findings are similar to previous studies that found that all of the tested isolates carried *speta* and *siet* [112,115,131]. The overall prevalence of *expA* carriage was 12% and *expB* carriage was 16%. Previous studies have reported a 0-6% *expA* carriage prevalence and 0-10% *expB* prevalence [112,115,131]. In this study, *expA* and *expB* were always carried in conjunction with *speta* and *siet*. There was no correlation between carriage of *expA* or *expB* and sex, age, or breed, and the proportion *expA* or *expB* – positive isolates was similar between healthy and diseased dogs. Carriage of ET genes was not linked to SCC*mec*, *spa*, or *dru* type. The most common *spa* type in the MRSP isolates was t06, as has been observed in the majority of *S. pseudintermedius* isolates cultured in North America [9]. The overall MRSP prevalence in the collection was 32%, and the most common SCC*mec*, *spa*, and *dru* type combinations were V+t06+dt11a. There was no correlation between methicillin resistance status and carriage of *expA* or *expB.* This is the first study to determine that odds of pyoderma were greater for dogs that were *expA*-positive when comparing healthy and diseased dogs.

Inflammation scoring study

The tissues examined included haired skin (n = 15), ear canal skin (n = 3), paw pad skin (n = 4), subcutis (n = 2), and unhaired skin (n = 1). The set included diseased dogs from the pyoderma (n = 6), surgical (n = 2), and other (n = 16) groups. There was a skin

biopsy from one dog in the healthy group that was evaluated for a small skin mass shortly after initial culture, and neoplasms were present in two additional cases. The majority (92%; 23/25) of the isolates in the paired cultures were MR. The carriage profiles for the paired biopsies included: *speta+siet* (n = 19), *speta+siet+expA* (n = 2), and *speta+siet+expB* (n = 4). The majority of the skin tissues were moderately inflamed (n = 10), with fewer cases of mild (n = 5), severe (n = 8), or minimal (n = 2) inflammation. More cases had mononuclear type inflammation (n = 11) than polymorphonuclear inflammation (n = 9) or a mixture of the inflammation types (n = 5). The ratio between superficial (n = 11) and deep (n = 12) inflammation was similar; however, the depth of inflammation could not be assessed in two cases due to the absence of identifiable epidermis or superficial skin structures in the biopsy. Bacteria were visible in nine of the cases, all having the *speta+siet* ET carriage profile.

The inflammation severity scores of the paired biopsies were correlated to the ET carriage profile of the associated bacterial isolates (P = 0.0002; FET) and to the type of inflammation (P= 0.0306; FET). The *speta+siet* profile was less likely to be associated with mild inflammation (P = 0.0001; two-tailed FET), and *speta+siet+expA* and *speta+siet+expB* were more likely to be associated with mild inflammation (P = 0.0333 and 0.0162, respectively; two-tailed FET) when compared to other severity categories. When grouping moderate and severe inflammation together, *speta+siet* was more likely to be associated with severity scores > 1 (P = 0.0022; two-tailed FET). Inflammation scored as mild was more likely to be mononuclear when compared to other severity or inflammation type groups (P = 0.0087; 2-tailed FET). The *speta+siet* profile was less

likely to be associated with mononuclear inflammation than other ET profiles (P = 0.0026; two-tailed FET), and *speta+siet+expB* was more likely to be associated with mononuclear inflammation (P = 0.0261; two-tailed FET). Depth of inflammation was correlated with the severity score (P = 0.0094; FET), with mild inflammation linked with a superficial distribution (P = 0.0373; two-tailed FET) and severe inflammation linked to a deep distribution (P = 0.0272; two-tailed FET). Inflammation depth was not correlated to ET carriage profile (P = 0.2880; FET), and methicillin-resistance status was not correlated with any of the examined histologic indices.

Drawbacks to the inflammation scoring study included the small sample size of only 25 out of the 475 dogs (5.3%) in the ET prevalence study that had paired biopsies to examine, paucity of the *expA* and *expB* carriage profiles, and uncertainty that the biopsies and cultures were taken from similar regions. The isolates associated with the paired biopsy set were almost exclusively MR, which could have also biased the results. Contrary to our hypothesis, the *speta+siet* profile was correlated with inflammation severity scores greater than 1 (moderate and severe) and the *speta+siet+expA* and/or *speta+siet+expB* carriage profiles were correlated with mild or mononuclear inflammation.

One explanation for these observations is that there are likely other virulence factors besides ET expressed in acute (i.e. predominately polymorphonuclear) skin lesions, and the tendency for *expA* and *expB* carrying isolates to be associated with mild or mononuclear inflammation suggests ET may have a role in chronic skin lesions. That mild inflammation is more correlated with mononuclear inflammation in our analyses may be due to differences in presentation of acute vs. chronic pyoderma. The correlations

between depth and severity of inflammation are expected as more severe lesions often encompass more tissue area and descend to deeper layers/structures within the skin.

CHAPTER IV

NATIVE INDUCTION OF *S. PSEUDINTERMEDIUS* EXFOLIATIVE TOXIN EXPRESSION AND ANTIBODY PRODUCTION IN DOGS

Introduction

Experiments with recombinant *S. pseudintermedius* EXPA have demonstrated that it degrades the extracellular domains of canine Dsg1, mouse Dsg1α, and swine Dsg1 but not degrade human Dsg1 or mouse Dsg1β and Dsg1γ [88]. Recombinant SIET does not degrade canine, mouse, human, or swine Dsg1 [88]. Recombinant EXPB degraded recombinant extracellular domains of canine Dsg1 but it has not been tested against Dsg1 from humans or other species [125]. SIET, EXPA, and EXPB do not degrade the deeper desmoglein-3 anchoring proteins [88]. The structural properties and target site of SPETA have not been determined [89]. Comparison of the predicted protein structures of the *S. pseudintermedius* SIET, EXPA, and EXPB proteins have revealed that the toxins have 78, 68, and 70% homology with SHETA, ETD and SHETB toxins, respectively, with lesser homology to ETA and ETB [89]. The protein structure of SPETA has not been evaluated. Based on the predicted target site of *S. pseudintermedius* ET and the homology to ET in other bacterial species, the *S. pseudintermedius* ET are presumed to be secreted outside the cell as exotoxins. While recombinant SIET, EXPA, and EXPB proteins have been made and expressed in *Escherichia coli* in other studies [88,124,125,134], none have looked at native ET protein expression in *S. pseudintermedius* isolates *in vitro*. Also, it is unknown if dogs with and without pyoderma produce antibodies to the *S.*

pseudintermedius ET. As the conditions that facilitate native expression of ET were unknown in *S. pseudintermedius*, we modeled our study on previous work with native ETA expression in *S. aureus* [135]. The expected molecular weights of recombinant EXPA and EXPB are 27.5 and 26.9 kDa, respectively [88,124,125]. The molecular weight of recombinant SIET has been reported as 30 [134] and 45 kDa [88]. The molecular weight of the synthetic GFP versions used in the Set 1 plasmids for this experiment are 27 (SIET and EXPB) and 30 kDa (EXPA), and the expected weights of the EXPA+GFP, EXPB+GFP recombinant proteins for Set 1 are 58, 54 kDa, and 57-72 kDa, respectively. Due to difficulties in purification of the Set 1 recombinant EXPA and EXPB (see Materials and Methods), the *expA* and *expB* genes were codon optimized and also cloned into another plasmid vector to facilitate protein purification (Set 2). The purpose of this experiment was to determine the conditions under which *S. pseudintermedius* isolates secrete SIET, EXPA, and EXPB, and to determine if dogs produce anti-SIET, -EXPA, and –EXPB antibodies.

Materials and methods

Production of primary anti-ET antibodies

In order to detect unlabeled, native ET proteins in culture supernatant, we contracted with LifeTein® LLC (Somerset, NJ) to produce affinity purified primary anti-SIET, -EXPA (EXI), and -EXPB antibodies. Two rabbits were immunized twice with synthetically produced peptide fragments representing the epitopes that were predicted to be the most antigenically stimulating, yet specific for each ET protein (**Table 2**). As

SPETA was present in every isolate tested and had few epitopes to target for optimal antibody production, we did not include this protein in the expression studies.

Protein	**Peptide Sequence**
SIET	ATSTVLEEGDSNKKIAEI-C
EXPA	C-KNVTTLGEDLKKRAKLQEE, includes a N-terminal KLH conjugation
EXPB	AKTYDEAEIIKKRDSFNTS-C

Cloning Primers (SIET and EXPB)	
Siet F (NdeI)- AAGGAGATATACATATGATGTCAAA AAGGATCATAGCG	**Siet R (NheI)-** GGTTTTCACCGCTAGCCCCTTCAT ATTTGTACATTGG
ExpB F (NdeI)- AAGGAGATATACATATGATGAATAA AACTACTTTTAAACAT	**ExpB R (NheI)-** GGTTTTCACCGCTAGCATCTTCTG ATTCAGCTCTTTT

Table 2: Synthetic peptide and cloning primer sequences for the Set1 SIET and EXPB proteins.

Recombinant ET protein control production

Recombinant ET proteins (Set 1 and Set 2) were made for use as controls for the native expression experiments. All plasmids used were pET expression vectors which utilized the T7 promoter for T7 RNA polymerase, encoded either ampicillin/carbenicillin (pET15) or kanamycin resistance (pET28), and allowed for induced expression of the proteins through addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to the media.

Set 1

Recombinant SIET and EXPB were made by cloning the native *S. pseudintermedius* DNA coding region (reference ADX76359.1 and AB569087.1, respectively) for each of the proteins into a pET15b backbone plasmid containing a C-terminal tobacco etch virus (TEV) protease cut site, fusion with superfolder green florescent protein (GFP), and a 6x His tag [136]. The plasmids were linearized with NdeI and NheI, and the ET gene inserts were amplified via PCR with 5' and 3' hanging ("sticky" ended) primers (Sigma-Aldrich, St. Louis, MO) containing the specific cut sequences for the restriction enzymes used (Table 2). The inserts were ligated using the In-Fusion® HD Cloning Kit (Clontech Laboratories, Inc.). Due to an intrinsic TEV protease cut site in EXPA, a pET backbone plasmid was designed and produced by VectorBuilder (Cyagen Biosciences Inc., Santa Clara, CA) containing an *E. coli* codon optimized open reading frame for EXPA (AB489850) within the multiple cloning site followed by a C-terminal enterokinase cleavage site, fusion to GFP, and a 6x His tag. The ligated SIET+GFP, EXPB+GFP, and EXPA+GFP plasmids were transformed into Novablue competent *E. coli* cells (Invitrogen) and plated onto 50 µg/mL carbenicillin plates. White colonies were selected and checked via PCR targeting the T7 promoter region for appropriate in-frame incorporation of the inserts based on Sanger sequencing (Eton Biosciences, Inc.).

Plasmids were purified using a Qiagen mini-prep plasmid kit using the standard protocol and were transformed into E. cloni® EXPRESS BL21(DE3) Chemically Competent Cells (Lucigen Corp., Middleton, WI). To induce recombinant ET expression, 20 mL cultures were grown until OD_{600} was 0.6 to 0.8, whereby 1 mM final concentration

(20 µL) of IPTG was added to the culture and the culture was incubated at room temperature overnight (12 h). The bacterial pellets were then lysed with BugBuster® Protein Extraction Reagent (EMD Millipore, Darmstadt, Germany) and the whole cell lysate was used in subsequent experiments to test anti-ET antibody specificity in the presence of bacterial extract from a non-staphylococcal species. Purification of the Set1 recombinant proteins using a nickel-charged affinity resin column (Ni-NTA Agarose; Thermo-Fisher Scientific) was unsuccessful, necessitating re-cloning and further expression experiments with EXPA and EXPB (see Set 2). Due to the results of some of the preliminary native ET expression experiments (see below), further purification of the recombinant SIET was not pursued.

Set 2

The coding DNA of *expA* and *expB* were codon optimized using the Codon Optimization Tool (http://www.idtdna.com/CodonOpt) and synthesized as gBlocks gene fragments (Integrated DNA Technologies, Coralville, Iowa). The synthesized *expA* and *expB* gene fragments were cloned into a pET28b-based vector (Agilent Technologies) with an N-terminal fusion with maltose binding protein (MBP), a 6x N-terminal His tag, and a TEV cleavage site between the 6xHisMBP tag and the ET protein sequences. The pET28b plasmids were linearized with BamHI and XhoI and the fragments were ligated into the plasmid backbone with T4 DNA ligase. The resulting plasmid was checked for proper, in-frame integration of the insert through DNA sequencing. The *E. coli* strain BL21Star (DE3) (Invitrogen) was co-transformed with the pET28b/6HisMBP-EXPA and

-EXPB expression plasmids and pLysSRARE2 plasmid (Agilent Technologies) and grown overnight in Terrific Broth (tryptone, yeast extract, and glycerol) containing chloramphenicol (34 µg/mL) and kanamycin (50 µg/mL) at 37ºC. The overnight culture was used to inoculate Terrific Broth containing kanamycin (50 µg/mL) and incubated while being shaken at 37 ºC until the OD_{610} reached 0.8. The culture was then chilled to 4 ºC, 1 mM of IPTG was added, and the culture was shaken for 24 hours at 22 ºC. Cells were harvested by centrifugation at 6000 × *g* for 10 min.

Purification of EXPA and EXPB from Set 2

The induced Set 2 MBP+EXPA and MBP+EXPB cell pellets were re-suspended in purification buffer (50 mM Tris/HCl, pH 8.0 containing 300 mM NaCl, 20 mM imidazole, and 10% glycerol). The homogeneous suspension was lysed with 4-5 passes through a M-110P Microfluidizer® (Microfluidics, Westwood, MA) at 20,000 psi, and then centrifuged at 4 ºC for 30 min at 30,000×g. The supernatant was then applied onto a HisTrap™ 5-mL column (GE Healthcare, Little Chalfont, United Kingdom), and eluted with purification buffer containing 500 mM imidazole, which was then removed by using a HiPrep™ 26/10 Desalting (GE Healthcare) on the ÄKTA pure chromatography system (GE Healthcare). The purified 6HisMBP-EXPA and -EXPB was then digested with TEV at a ratio of 6.67 µg of TEV per mg of recombinant protein overnight at 4 ºC, and was then loaded onto a HisTrap™ 5-mL column; the flow-through was harvested and concentrated, and then was loaded onto a HiLoad® 16/600 Superdex® 200 pg column (GE Healthcare) which was pre-equilibrated with size-exclusion buffer (TBS, pH 7.4) for

gel filtration on the ÄKTA pure system. The fractions containing tag-free EXPA and EXPB were pooled and concentrated by using Amicon Ultra-15 Centrifugal Filters with a 10,000 Da nominal molecular weight limit (Merck Millipore, Billerica, MA). The purity of the tag-free EXPA and EXPB was checked via 4-20% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The protein concentration was determined by DC Protein Assay with a Bio-Rad (Hercules, CA) kit, using bovine serum albumin as the standard.

Antibody specificity experiments

The specificity of the anti-ET antibodies was tested via dot blot and Western blot using the synthetic peptides provided by LifeTein® LLC and the *E. coli* recombinant ET proteins from Set 1 and 2 as positive controls. Samples were tested with nitrocellulose and polyvinylidene difluoride (PVDF) membrane of 0.45 and 0.2 µM pore sizes. Lyophilized SIET, EXPA, and EXPB synthetic peptides were diluted in phosphate buffered saline (pH 7.0) and either dotted onto nitrocellulose membrane (pore size 0.2 µM) or transferred to a PVDF (pore size 20 µM) membrane for Western Blot. For Western Blot, samples (peptides, bacterial lysate from Set 1, and purified Set 2 recombinant proteins) were mixed 1:1 with 2X Tris-glycine-SDS loading buffer and heated to 85°C for 10 min. EXPA and EXPA peptide controls were only heated briefly (< 1 min) to avoid complete proteolysis of the small fragments and SIET peptide was not heated at all. The samples were loaded onto a NuPAGE™ 4-12% Bis-Tris, 1 mm-thick protein gel (Thermo-Fisher) and electrophoresed in 1X 3-(N-morpholino) propanesulfonic acid (MOPS) buffer at 170V for

60 min in an XCell SureLock™ Mini-Cell Electrophoresis System (Thermo-Fisher Scientific). The proteins were transferred from the gel onto prepared PVDF membrane in chilled Towbin buffer at 100V for 60 min with a Mini-PROTEAN® electrophoresis system (Bio-Rad). The PVDF membrane was blocked for 1 h with TTBS buffer (50 mM Tris-HCL (pH 7.4), 150 mM NaCl, and 0.1% Tween 20) + 5% powdered non-fat skim milk. Primary antibody was added at a dilution of 1:5500 in TTBS + 5% non-fat skim milk buffer and incubated with gentle rocking at 4°C for 12-14 h. The membrane was washed with TTBS three times at 15 min, and secondary goat anti-rabbit IgG horseradish peroxidase conjugated antibody (31460; Thermo-Fisher Scientific) was added at a 1: 200,000 dilution and incubated at room temperature for 1 h. The membrane was washed in TTBS three times for 10 min before 0.5 mL each of SuperSignal™ West Pico Chemiluminescent Substrate (Thermo-Fisher) parts A and B were added and incubated with agitation for 5 min. Membranes were wrapped in cellophane wrap and exposed to x-ray film for 30 min to 1 hr. For dot blots, 3-5 µL of sample was dotted onto either nitrocellulose or prepared PVDF membrane and left to dry for 1 hr before blocking and processing as described for the Western blots. The controls (**Fig. 3**) demonstrated bands of expected size for the peptides or fusion with GFP on Western blot (individually tested and in combination with all three anti-ET antibodies).

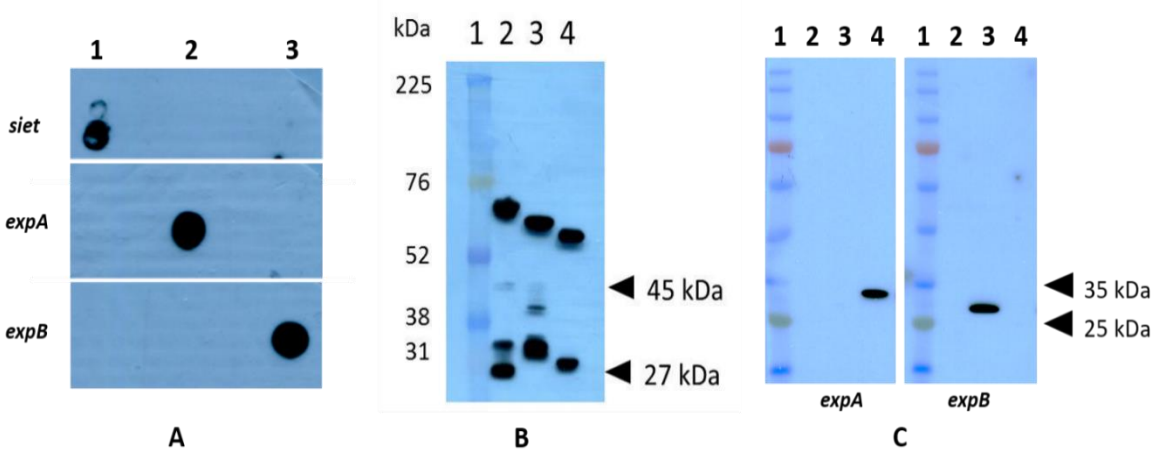

Figure 3: Peptide and recombinant ET protein controls.
A: Dot blot on nitrocellulose (0.2 µM) membrane of ET peptides using anti-SIET (top), EXPA (middle), and EXPB (bottom) antibodies. 1 = SIET, 2 = EXPA, and 3 = EXPB
B: Western blot on PVDF (0.2 µM) membrane of Set 1 recombinant GFP+ET proteins (50 ng lysate loaded) using anti-SIET, EXPA, and EXPB antibody cocktail. The larger bands represent fusion of GFP with the ET proteins and the smaller bands are ET protein separated from GFP. 1 = Molecular weight marker (GE Healthcare; RN756E), 2 = GFP+SIET, 3 = GFP+EXPA, 4 = GFP + EXPB
C: Western blots on PVDF (0.2 µM) membrane of Set 2 purified, untagged EXPA and EXPB (20 ng loaded) with anti-EXPA (left) and -EXPB (right) antibodies. 1 = Molecular weight marker (SMBio; PM2500), 2 = LB broth blank, 3 = EXPB, 4 = EXPA

Induction of native ET production in select *S. pseudintermedius* isolates

Isolates representing each of the ET carriage combinations were tested (i.e. *speta*-only, *speta+siet*, *speta+siet+expA*, etc.). The following represents the protocol that produced the most consistent expression of ET, although other permutations were tested. A single colony was inoculated into 1 mL of full salt lysogeny (i.e. Luria-Bertani) broth in a 5 mL glass test tube with a side-ventilated plastic cap that was loosely fitted. The cultures were incubated with 10% CO_2 and agitation on a 2D orbital shaker at 200 rpm in a water jacketed incubator with water pan (Forma Scientific; Thermo-Fisher). The

incubator was initially at room temperature (22 °C) and slowly warmed to 37°C over the course of 2 h; the cultures were incubated for 12-14 h. The bacterial culture was pelleted by centrifugation at 5,510 x *g* for 15 min, the supernatant was removed and filtered with a 0.45 µm syringe filter, and the supernatant was chilled for 1-2 h at 4°C. Ice-cold trichloroacetic acid (TCA) 6.1 N solution (250 µL) was added to each reaction and the samples were cooled at 4°C for 1 hr. Samples were centrifuged in 1.5 mL conical Eppendorf tubes at 14,000 x *g* at 4°C for 20 min. The TCA supernatant was removed using wide-mouth 1 mL pipette tips, the protein pellets were re-suspended in ice cold acetone two times, and the mixture was centrifuged at 14,000 x *g*, at 4°C for 10 min. The pellets were dried for 10 min at 80 °C before resuspension in 30 µL of phosphate buffered saline (pH 7.0). Concentrated supernatant was used for dot blot or Western blot as described for the antibody specificity experiments, except 50 ng of Set 1 bacterial recombinant protein lysates or 20-50 ng of purified EXPA and EXPB protein were used as controls and the blots were exposed to x-ray film for up to 2 hours to optimize exposure due to the low concentration of ET protein in the native expression samples. Paired gels were also stained with Coomassie blue to visualize all of the protein bands in the samples used for the Western blots.

Antibody production to EXPA and EXPB in dogs

Serum from thirteen dogs with a clinical history of pyoderma or history of culture of *S. pseudintermedius* (pyoderma/exposure group) and thirteen dogs that presented for other was conditions obtained for the study (control group). Dogs in the control group

presented for orthopedic (n = 4), urinary (2), neurologic (2), hepatic (1), neoplastic (2), endocrine (1), and cardiovascular (1) conditions. All serum samples were residual diagnostic specimens and were collected under the ethical and standards of care guidelines for the Texas A&M Veterinary Medical Teaching Hospital (VMTH). The criteria for inclusion in the pyoderma/exposed group were either mention of dermatitis, pyoderma or allergic skin disease in the major problem list in the clinical record (n = 10) or culture of *S. pseudintermedius* from the skin (n = 3) or another body site (n = 3; one each from cornea, lung, and ear). Two dogs in the pyoderma/exposure group contributed isolates in the ET prevalence study (*speta+siet* and *speta+siet+expB* phenotypes). ET protein control samples (1 µg of Set 2 purified EXPA and EXPB and 1 µg of Set 1 GFP+SIET lysate) were heat denatured and run on a NuPAGE™ 4-12% Bis-Tris, 1 mm-thick protein gel (Thermo-Fisher) and processed for Western blot as described in the antibody specificity experiment methods. A gel with the samples was also stained with Coomassie Brilliant Blue. The EXPA and EXPB purified protein did contain a small amount of the MBP tag (45 kDa) that was visible on the Coomassie stained gel (see below) and served as a negative protein control in addition to an LB blank in one lane. Pooled pyoderma/exposure and control serum samples containing 100 µl of serum from individual dogs in the groups were used as the primary antibodies and were diluted 1:1,000 in TTBS, and the PVDF blots were incubated at 4°C overnight (12 h) with gentle rocking. The blots were washed in TTBS 3x for 15 min and then incubated with secondary goat anti-canine IgG antibody conjugated with HRP (A18763; Thermo-Fisher Scientific) at 1:10,000 dilution at 22°C for 60 min. The blots were washed 3x in TTBS for 10 min before 0.5 mL each of

SuperSignal™ West Pico Chemiluminescent Substrate (Thermo-Fisher) parts A and B were added and incubated with agitation for 5 min. Membranes were wrapped in cellophane wrap and exposed to x-ray film for up to 3 hr.

Results and discussion

Native ET induction

Expression of EXPB and EXPA was observed for the single and dual carriage isolates with bands that corresponded to the predicted sizes for each of the proteins. SIET was only labeled in the synthetic peptide controls on nitrocellulose dot blot and in the recombinant SIET+GFP protein, and native expression of SIET was not observed in the tested *S. pseudintermedius* samples (results summarized in **Fig. 4**) using a protocol that induced expression of EXPA and EXPB. Two faint bands were seen on the SIET blots in *speta +siet + expA* and *speta + siet +expB* carriage isolates at approximately 20 kDA, but this product is smaller than the predicted size of SIET and likely represents background staining. The SIET peptide degraded quickly and did not transfer well to PVDF membrane for Western blot; interestingly, the native induction and lysed bacterial Set 1 control samples did not transfer well to nitrocellulose membrane of either pore size, and were instead best observed on PVDF. This difference was most likely due to buffer differences in the lysis and resuspension buffers of the samples. While exact quantification of the amount of EXPA and EXPB secreted into the supernatant could not be performed due to the need to concentrate the protein to visualize on the Western blot, the amount of signal achieved in the bacterial samples was comparable to the amount of signal achieved when

20 ng of the purified protein controls were loaded onto the gel. This finding suggests that EXPA and EXPB production can be induced in *S. pseudintermedius* and at least 20 ng of the ETs can be produced per 1 mL of bacterial supernatant under the experimental induction conditions outlined in this experiment. This study represents the first time native expression of ET has been attempted in *S. pseudintermedius* and suggests that EXPA and EXPB are secreted in significant amounts outside of the bacterial cell.

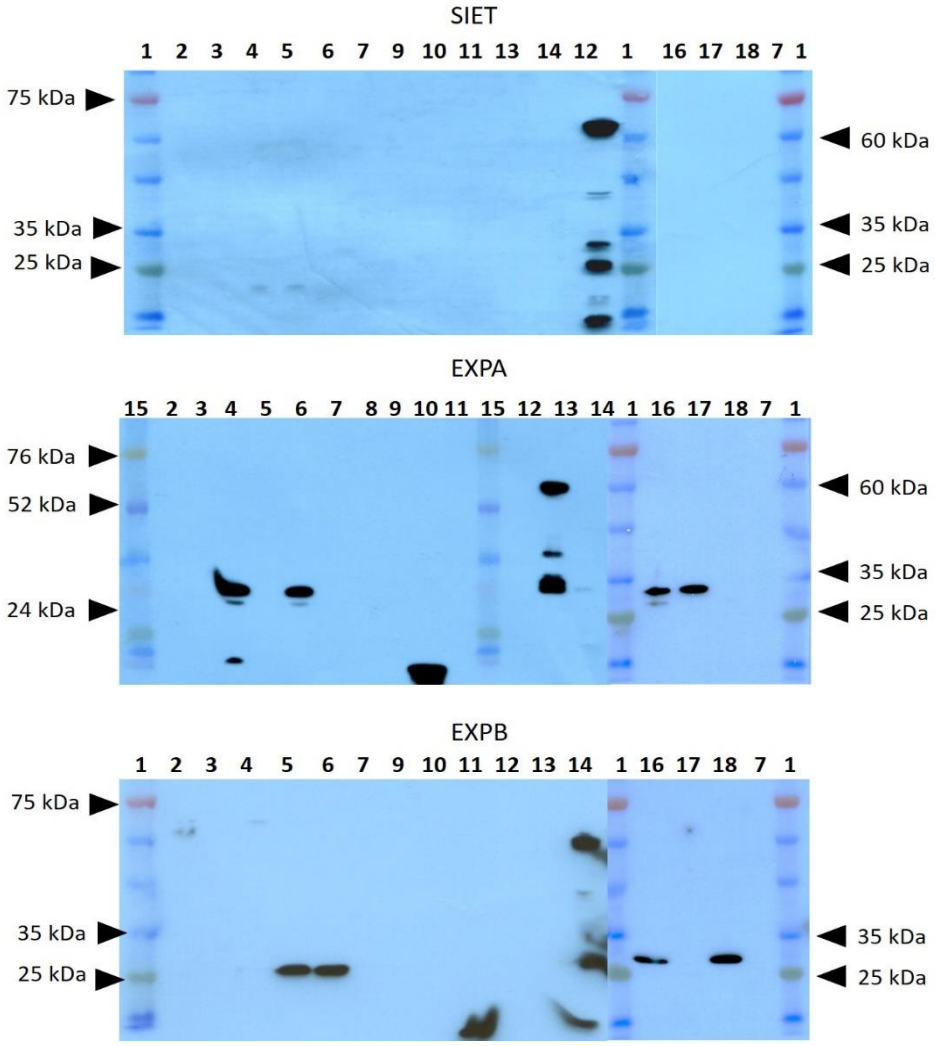

Figure 4: Native induction of SIET (top), EXPA (middle) and EXPB (bottom) in *S. pseudintermedius* isolates. Western blots of *S. pseudintermedius* samples and controls.

1 = Protein Molecular weight marker (SMOBio; PM2500)
2 = *speta* only (12-017)
3 = *speta* + *siet* (19-040)
4 = *speta* + *siet* + *expA* (37-029)
5 = *speta* + *siet* + *expB* (19-008)
6 = *speta* + *siet* + *expA* + *expB* (33-100)
7 = LB blank
8 = dH_2O
9 = SIET peptide
10 = EXPA peptide
11 = EXPB peptide
12 = Set 1 pET15+SIET+GFP+6xHis (bacterial lysate)
13 = Set 1 pET15+EXPA+GFP+6xHis (bacterial lysate)
14 = Set 1 pET15+EXPB+GFP+6xHis (bacterial lysate)
15 = Protein molecular weight marker (GE Healthcare; RN756E)
16 = *speta* + *siet* + *expA* + *expB* (29-002)
17 = Set2 purified EXPA (20 ng untagged)
18 = Set2 purified EXPB (20 ng untagged)

Canine serum samples

Sera from the pyoderma/exposed and control dog groups contained antibodies to purified Set2 EXPA and EXPB (**Fig. 5**), with antibodies in the pyoderma group also binding to a band of the approximate size of the recombinant SIET+GFP from Set 1 as well as multiple proteins in the *E. coli* bacterial lysate. The band intensity was greater in sera from the pyoderma/exposure group when compared to sera from the control group, but there was still appreciable binding of antibody to the EXPA and EXPB proteins in the control group. This finding suggests that dogs without a reported history of clinical pyoderma were also exposed to and produced antibodies to ET. The greater intensity of binding to the EXPB protein in the pyoderma group could be explained by the one individual in that group with known exposure to EXPB based on the prevalence study data. Other considerations are that the clinical histories of the dogs in the control group could have been incomplete or a few individuals previously had clinical or subclinical pyoderma or the dogs were exposed to *S. pseudintermedius* ET via another route (i.e. mucous membrane exposure and/or ingestion). These results show that dogs can produce an antibody response to EXPA and EXPB, and dogs with a history of pyoderma/ exposure to *S. pseudintermedius* may also produce antibodies against SIET.

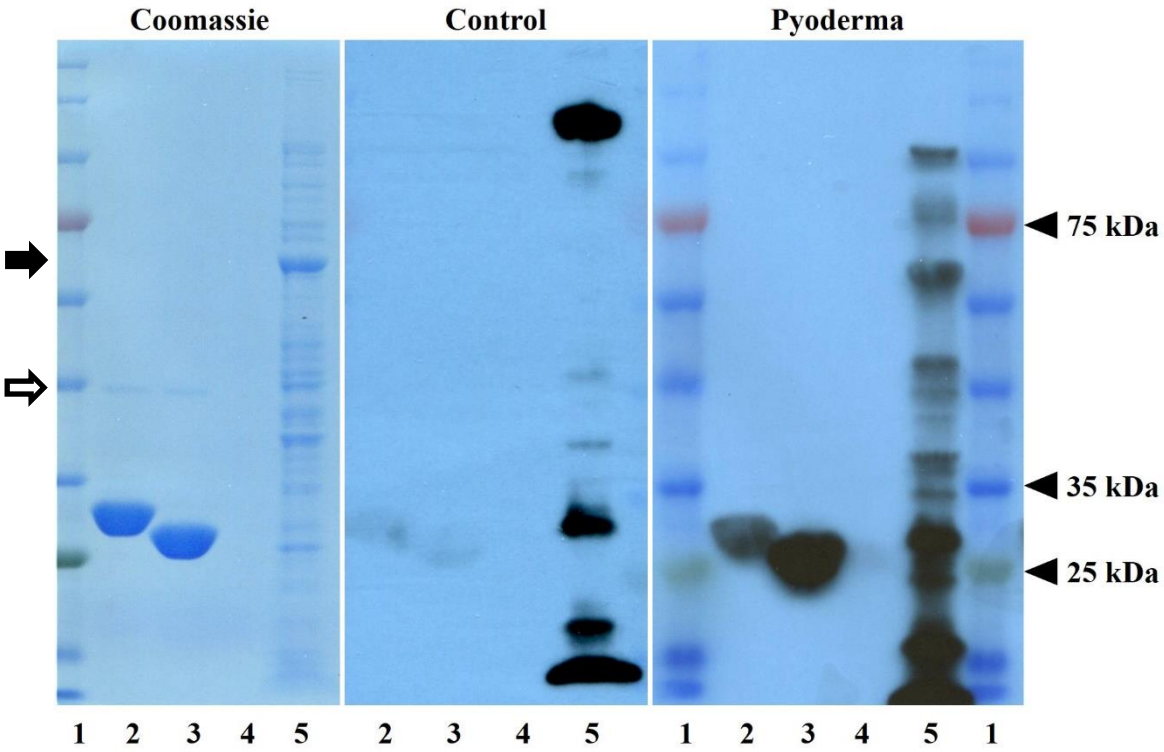

Figure 5: Antibody production against ET in dogs. Western blot using sera from dogs without a history of pyoderma (middle) and dogs with a history of pyoderma or culture of *S. pseudintermedius* (right). The black arrow indicates the approximate size of the SIET+GFP band in the induced *E. coli* bacterial lysate on the Coomassie Brilliant Blue stained SDS-PAGE gel (left). The MBP tag is indicated by the white arrow in lanes 2 and 3 in the Coomassie gel, which the canine sera antibodies did not bind to in either test group.

1 = Protein Molecular weight marker (SMOBio; PM2500)
2 = Set 2 purified EXPA (untagged, 1 µg)
3 = Set 2 purified EXPB (untagged, 1 µg)
4 = LB blank
5 = Set 1 pET15+SIET+GFP+6xHis (bacterial lysate)

CHAPTER V

EXFOLIATIVE TOXIN GENE MAPPING IN *S. PSEUDINTERMEDIUS* ISOLATES

Introduction

Virulence genes in other species of staphylococci are frequently carried on MGE that are transferred through plasmids, transposable elements, or phages that facilitate horizontal transfer of virulence factor genes between bacteria and contribute to the development of antimicrobial resistance [83]. For instance, *S. aureus* ETB and *S. hyicus* SHETB are both encoded on plasmids, and other staphylococcal exotoxins, such as ETA, are carried on phage [83]. ET gene expression is controlled by regulators, such as the *agr* regulatory group, which influences expression of ETA and ETB in *S. aureus* [137]. In previous studies in *S. aureus*, ET producing strains have been closely related and are usually linked with the *agr* type IV allele [80], but it is not known if *agr* allele type differs among ET-carrying strains of *S. pseudintermedius*.

Previous studies with the *siet*, *expA*, and *expB* genes have determined that they are not carried on plasmids [88,125]. Three of the four purported ET genes are found within the *S. pseudintermedius* reference genomes within GenBank. The *siet* gene is positioned on the chromosome between the transmembrane protein YfcA and a thiol peroxidase. The *speta* gene is adjacent to a phage encoded chromosome degrading nuclease YokF (also known as uncharacterized protein YhjA; position 68,188- 69,103 in HKU10-03 reference genome). Both *speta* and *siet* are consistently located in these regions on the chromosome in the 18 *S. pseudintermedius* reference genomes available at GenBank and do not appear

to be associated with MGE. The only sequence giving any context to the position of *expA* within the *S. pseudintermedius* chromosome is a partial sequence in the incomplete, unindexed 2001-08-299-1 strain draft genome (ASM162294v1). The *expB* gene is not present within the reference genomes at GenBank and has not been mapped. It is unknown if *expA* or *expB* are located on MGE that may facilitate horizontal transmission of the genes. The purpose of this analysis was to map the *expA* and *expB* genes within the chromosome and to determine if they were associated with MGE. The MLST and *agrD* auto-inducible protein sequence typing schemes developed for *S. pseudintermedius* [71,138] were also determined for the 13 examined isolates.

Materials and methods

Bacterial culture and DNA extraction

Two isolates that carried *expA* and *expB*, one isolate that carried *speta + siet*, and five isolates that carried either *expA* or *expB* were chosen for sequencing (**Table 3**). The isolates were struck onto blood agar from the freezer stock vials (LB with 20% glycerol) and incubated at 37 °C for 12 h; the culture was sub-cultured onto another blood agar plate. Finally, a single colony was used to inoculate 1 mL of brain-heart infusion broth (BHI) and the culture was incubated at 37 °C for 12 h on an orbital shaker set at 200 rpm. The cultures were transferred to 1.5 mL, plastic conical bottom Eppendorf tubes, and the bacteria were pelleted by centrifugation at 6000×g for 10 min and the supernatant was removed. DNA was extracted using a MasterPure™ Gram Positive DNA Purification Kit (Epicentre; Madison, WI) according to the manufacturer's recommendations for *S. aureus*

DNA extraction, except the samples were incubated at 37 °C for 12 h at the lysis buffer step and the DNA pellets were resuspended in 30-60 µL of elution buffer at the elution step.

ET gene profile	Isolate #	Methicillin resistance status	*Spa* type	Group
expA	29-095	MS	NA	Urine
	30-085	MS	NA	Healthy
	38-056	MS	t06	Pyoderma
	37-029	MS	t06	Pyoderma
	41-073	MS	t06	Surgical
expB	12-005	MS	t05	Surgical
	30-090	MS	t76	Pyoderma
	31-062	MS	t07	Pyoderma
	37-024	MS	NA	Urine
	39-026	MR	NA	Pyoderma
expA + *expB*	29-002	MS	NA	Healthy
	33-100	MS	t06	Urine
speta + *siet*	19-040	MS	t02	Urine

Table 3: Characteristics of 13 *S. pseudintermedius* isolates used for WGS. MR = methicillin-resistant, MS = methicillin sensitive.

Whole genome sequencing

The recommended minimum depth of sequencing required for *de novo* assembly of *Escherichia coli* genomes is 50-fold, and this coverage benchmark is recommended for WGS of other small bacterial genomes as higher magnitude coverage can result in pseudogene amplification within runs [139]. Sequencing libraries were prepared using the NEXTflex® Rapid DNA-Seq Library Prep Kit for Illumina® Sequencing (Bioo Scientific, Austin, TX). DNA was quantified using the Qubit™ 2.0 (Life Technologies, Carlsbad,

CA) high sensitivity double stranded DNA (dsDNA) assay and all samples were normalized to 100 ng of DNA in a total volume of 14 µL. Five microliters of normalized DNA (1 ng total DNA) was used for library preparation. Prepared libraries were checked with the Qubit™ 2.0 dsDNA assay to determine concentration and a TapeStation D1000 HS (Agilent Technologies, Santa Clara, CA) was used to determine the average fragment size of the prepared libraries. All samples were normalized to 4 nM and all samples were pooled together for sequencing. The 4 nM pool was diluted to 20 pM and sequenced on the Illumina® MiSeq 300 x 300 cycle v3 sequencing kit. All run data and FASTQ files were uploaded to BaseSpace (https://basespace.illumina.com/) for downstream analysis.

Genome assembly and annotation

The Illumina® MiSeq paired read libraries for each isolate were *de novo* assembled using the careful mismatch correction option in SPAdes (v. 3.6.2). The online sequencing pipeline offered by the Pathosystems Resource Integration Center (PATRIC version 3.4.6; https://www.patricbrc.org) was used to annotate the genomes [140]; annotation was done using the Rapid Annotation using Subsystem Technology tool kit (RASTtk) on the PATRIC pipeline [141].

Analysis of ET gene position and identification of MGE

The SPETA (ADX77621), SIET (CAR57917.1), EXPA (BAI49625.1), and EXPB (BAJ23893.1) amino acid sequences available at UniProt (http://www.uniprot.org/) were queried against the NCBI reference database using the protein BLAST available in the

PATRIC interface. The nucleotide and amino acid sequences for *expA* and *expB* in respective isolates were compared using the multi-sequence alignment tool in PATRIC. The locations of all four ET genes within the annotated genomes were analyzed with the Compare Region Viewer in PATRIC, and the ET gene regions were compared with the *S. pseudintermedius* reference genome HKU10-03 and amongst the different ET carriage groups. The contigs containing EXPA and EXPB in the sequenced isolates were analyzed with the online resources PHAST and PlasmidFinder 1.3 to identify prophage elements and plasmid origins of replication, respectively [142,143]. To find pathogenicity island (PI) components, contigs containing ET genes were queried using the online Pathogenicity Island Database (PAI DB version 2.0; http://www.paidb.re.kr/search_blast.php) [144].

MLST, *agr* determination, and presence of selected virulence genes

MLST was assigned *in silico* based on the previously described method using the assembly files for selected isolates and the batch query function of the online BIGSdb genomics MLST platform for *S. pseudintermedius* [71,78]. New types were submitted for inclusion in the database. Grouping into CC was performed with the eBURST algorithm in the BIGSdb database using all *S. pseudintermedius* isolates deposited in the database as of October 2017. Sequences for SEA, SEB, SED, and TSST-1 available in the staphylococcal VirulenceFinder 1.5 database files provided by the Center for Genomic Epidemiology (current as of March 2017) were used as reference sequences for the PATRIC BLAST queries [145]. The *agr* type was assigned by PATRIC BLAST query of the amino acid motifs unique to each type: I- RIPTSTGFF, II- KIPTSTGFF, III-

RIPISTGFF, and IV- KYPTSTGFF [114]. The sequences reported for LukSF-I [99], *sec*$_{canine}$, *se-int* [109,110], and BacSp222 bacteriocin [100] were also queried.

Results and discussion

ET gene mapping

Speta and siet

There was one significant single nucleotide mutation in the *speta* gene shared by three isolates (38-056, 30-090, and 39-026) that resulted in a phenylalanine to leucine amino acid substitution at position 294. There were two mutations in the *siet* gene that resulted in missense mutations in the protein sequence: in two isolates 37-029 and 30-090, there was a single nucleotide substitution that resulted in an alanine to threonine amino acid substitution at position 79, and in seven isolates there was an arginine to lysine substitution at position 185. The consequences of these mutations on the function of SPETA and SIET warrants further study as the substituted amino acids are of a different functional class than the replaced amino acid in the reference sequence. The *speta* gene was consistently downstream of the *yhjA* protein gene (**Fig. 6A**) and a lipase precursor, but the genes flanking the downstream region of *speta* were variable. In one of the *expB* isolates (31-062), the downstream region to *speta* contained several proteins associated with 70-90% homology to PI proteins in *S. aureus* SaPIbov (integrase- AF217235) and SaPI1 (terminase- U93688). The isolate 33-100 also had an identical integrase protein to the one found in 31-062. Carriage of the *speta* gene does not appear to be associated with

these elements as *speta* occurs in the same position in the genome regardless of concurrent carriage of the predicted PI-associated proteins.

The *siet* gene was flanked by the same genes (**Fig. 6B**) in all of the *S. pseudintermedius* genomes sequenced, and the position between transmembrane protein *YfcA* and a thiol peroxidase was the same as reported in the HKU10-03 reference genome. No plasmid replication, phage-like proteins, or proteins associated with PIs were identified in regions of the contigs upstream or downstream of the *siet* gene in all of the sequenced isolates and reference genome.

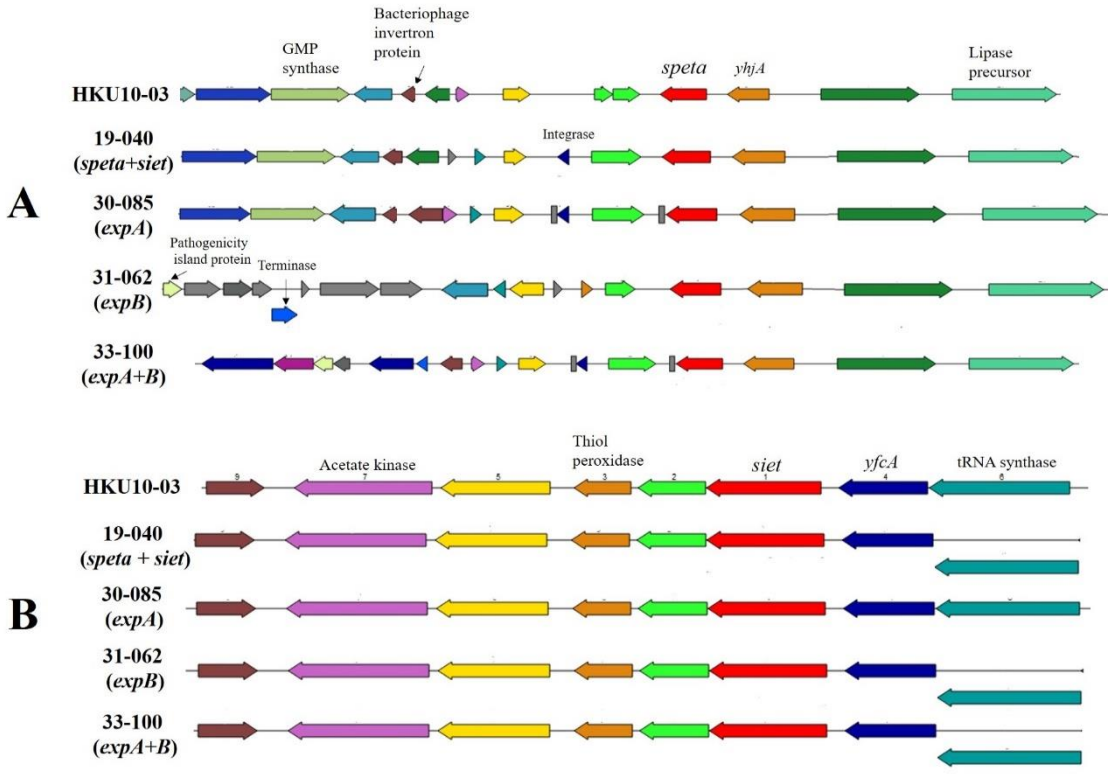

Figure 6: Position of *speta* (A) and *siet* (B) in selected genomes of *S. pseudintermedius*. Selected genes are labeled as annotated in PATRIC and the same color indicates the same gene in each line. The positions of *speta* and *siet* are indicated in red. The arrows indicate the direction of the open reading frame of the gene (i.e. 5' to 3' direction). HKU10-03 is the *S. pseudintermedius* reference genome.

ExpA

The nucleotide and amino acid sequences for *expA* were identical in the single and dual carriage groups to the *expA* reference sequence (AB489850). The *expA* sequence in all of the examined isolates was adjacent to a reverse transcriptase retroelement (annotated as a retron-type RNA-directed DNA polymerase) that contained numerous tRNA repeat regions and an uncharacterized mobile element protein, and *expA* was downstream of recombination and repair protein (RecF), DNA gyrase subunits, and threonine dehydratase. A NCBI BLAST-p search of the retroelement protein sequence revealed that the element has 95% identity with a Group II intron reported in *S. pseudintermedius* as opposed to a true retron-type system. Group II introns are MGE that encode reverse transcriptase and are the only retroelements capable of non-GTP-mediated intron excision (i.e. autonomous mobility) [146]. Group II introns are rarely observed in staphylococcal genomes, but one to two copies have been identified in the HKU10-03 *S. pseudintermedius* reference genome [147]. In previous cases with other species of bacteria, insertion of Group II introns has not been associated with the presence of specific genes, but they may influence expression of downstream genes or trigger recombination events of genes within adjacent regions [148]. Interestingly, identical group II intron-like sequences were adjacent to *expA* in all of the genomes that carried *expA*, suggesting that this retroelement has an important role in transfer of *expA*. In six of the isolates, only the region upstream to *expA* was completely sequenced which is most likely caused by poor alignment within the repetitive elements in the retron RNA domain sequence, a frequently observed problem with short-read WGS methods. The only sequence containing the region

downstream of *expA* was in isolate 29-002. However, BLAST search of other elements in the 29-002 sequence revealed identical regions in the genomes of the other isolates carrying *expA*, which suggests that the sequence in 29-002 is representative of the region downstream of *expA*. PHAST queries of the upstream and downstream regions to *expA* did not reveal any components that were similar to known phage-related proteins or elements, and a PlasmidFinder query did not reveal any plasmid origins of replication in the areas. A PAI DB BLAST search of the downstream 29-002 sequence revealed an integrase-like protein with 82% sequence identity with an unnamed integrase protein (AAG29618.1) in *S. aureus* PI SaPIbov; no elements in the upstream *expA* sequences in the other *expA* carrying isolates returned hits via PAI DB BLAST query. Further investigation is needed to bridge the areas of the genome flanking the *expA* gene (**Fig. 7**) and to determine if the Group II intron or integrase sites may influence excision or insertion of *expA* in the genome. This could be accomplished by utilizing long-read WGS methods (i.e. nanopore sequencing) or Sanger sequencing to resolve the repetitive regions and join the predicted upstream and downstream sequences.

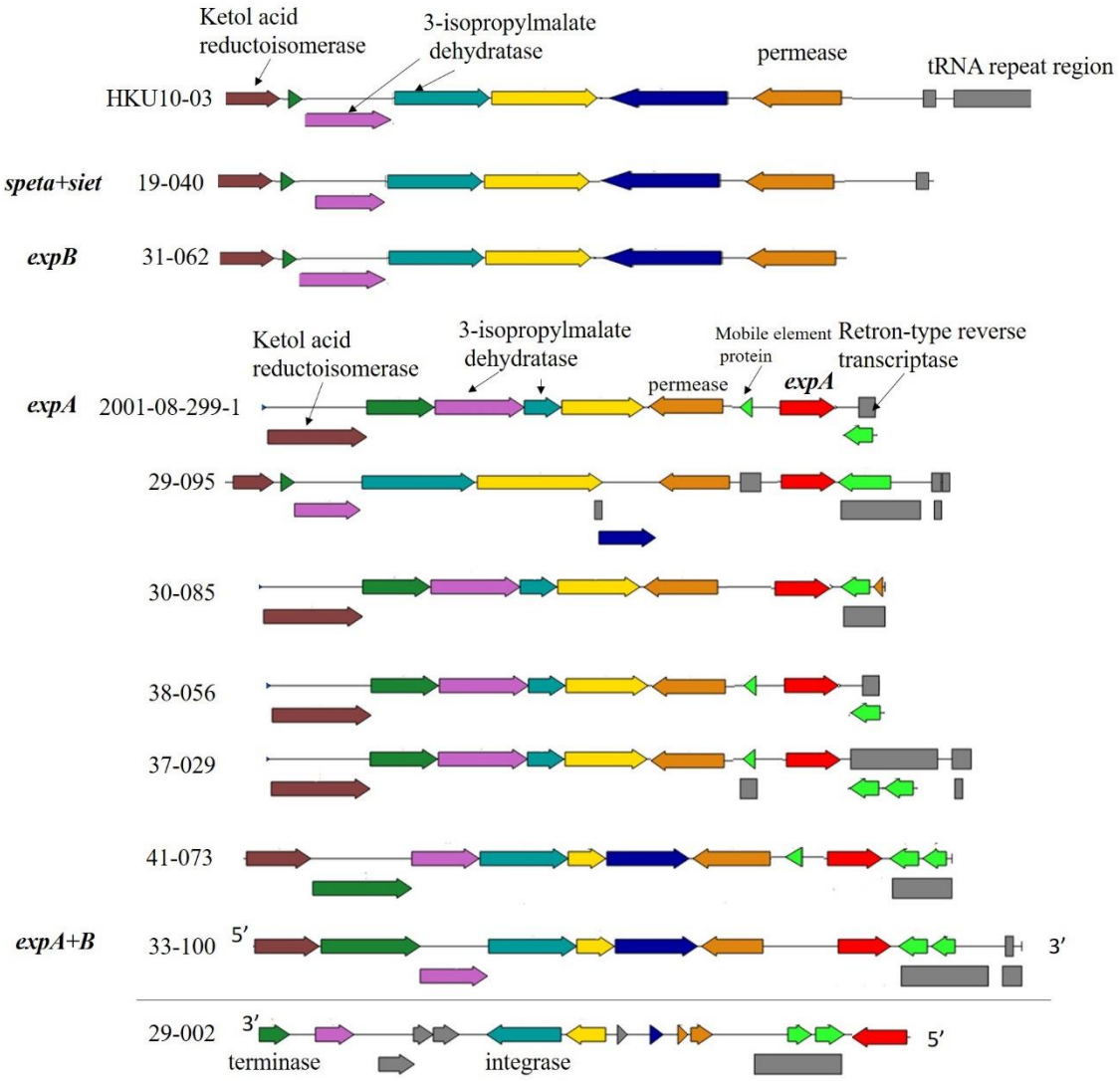

Figure 7: Position of *expA* in isolates with different ET gene carriage profiles. The *expA* gene (red arrow) was located upstream of a retron-type reverse transcriptase. Selected genes are labeled as annotated in PATRIC and the same color indicates the same gene in each line, except for the 29-002 downstream sequence which is given in the 3' to 5' direction. The downstream region to *expA* was only captured in isolate 29-002. The arrows indicate the direction of the open reading frame of the gene (i.e. 5' to 3' direction). HKU10-03 is the *S. pseudintermedius* reference genome. Isolate 2001-08-299-1 is a publically available draft genome that carries *expA*.

ExpB

The mapping findings for *expB* in the sequenced isolates are summarized in **Fig. 8**. There was variation in the *expB* amino acid sequence in two (12-005 and 30-090) of the examined single carriage (*speta+siet+expB*) and one (29-002) of the dual (*speta+siet+expA+expB*) isolates from the *expB* reference sequence (AB569087). In these isolates, there was an A to G base substitution at nucleotide position 70, resulting in an arginine to glycine amino acid substitution at codon position 24. The impact of the Arg-Gly substitution on the function of *expB* in these isolates warrants further study. The remaining three single carriage and one of the dual carriage isolates had 100% identity to the reference sequence. The *expB* gene is adjacent and upstream of the *se-int* exotoxin gene, fosfomycin resistance protein (*fosB*) and β-lactam resistance protein (*fmtA*). There was fragmented, incomplete coverage of the *expB* region in 29-002, but the isolate did have some of the same components in this region as observed in the other dual carriage isolate 33-100 and the single *expB* carriage isolates. PHAST queries of the upstream and downstream regions to *expB* did not reveal any components that were similar to known phage-related proteins or elements in the database, and a PlasmidFinder query did not reveal any plasmid origins of replication in the *expB* flanking regions. A prophage integration site was upstream of *se-int* in HKU10-03. The proteins annotated as PI-related proteins by PATRIC in the two *expB* configurations (33-100 and 12-005 as examples) were analyzed with PAI DB and revealed a potential transposase/phage integrase (32% identity with IS1133 transposase) and a MGE with characteristics of a transcriptase in both configurations; an uncharacterized PI protein (52% identity with phage-associated protein

in SaPIbov2) was identified in isolates with a configuration similar to 33-100. The findings suggest that the mobile element protein and the predicted transposase/integrase may be important in the carriage and potential horizontal transfer of *expB*.

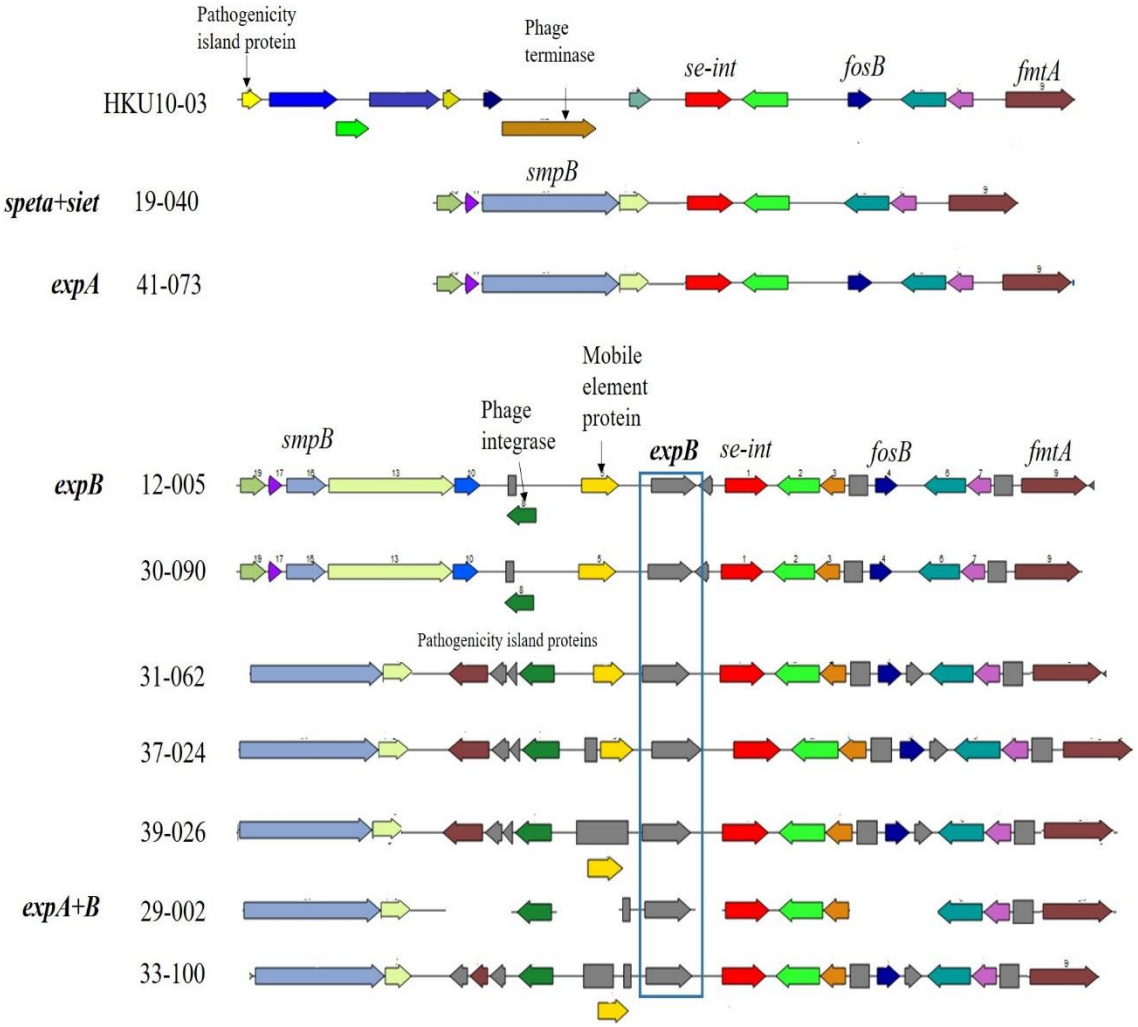

Figure 8: Position of *expB* in isolates with different ET gene carriage profiles.
The *expB* gene (gray arrow in blue box) was located upstream of the *se-int* exotoxin gene. The *expB* region in isolate 29-002 was highly fragmented. Selected genes are labeled as annotated in PATRIC and the same color indicates the same gene in each line. The arrows indicate the direction of the open reading frame of the gene (i.e. 5' to 3' direction). HKU10-03 is the *S. pseudintermedius* reference genome.

MLST and virulence gene carriage characteristics

The MLST, *agr* type, and other selected virulence gene carriage findings are summarized in **Table 4**. Nine new MLST were identified (ST755, 762, 765-771). Two of the *expB* isolates, 30-090 and 37-024, carried *ack* gene allele types (designated MLST New1 and New2) that were not recorded in the BIGSdb. All of the isolates had different ST and were members of four different CC. For seven of the isolates, the CC was unable to be determined, either because the isolate could not be placed in a group (i.e. a singleton), possessed a novel allele type, or was in a group where the founder could be determined due to paucity of isolates in the group. The *agr* types encountered were diverse for each ET gene carriage profile and included type II (n = 6), IV (4), and III (3) systems. None of the isolates carried BacSp222 or genes with > 90% homology to *S. aureus* SEA, SEB, SED, or TSST-1. The absence of carriage of BacSp222 in the small sample set is unsurprising as carriage of this bacteriocidin is rare [100]. All of the examined isolates carried *lukSF-I* and *se-int*, findings that are similar to the almost universal carriage mentioned in previous reports [99,109,112]. Two isolates of differing ET gene carriage profile carried sec_{canine} (41-073 and 29-002). Previous studies with canine-derived *S. pseudintermedius* isolates have reported a sec_{canine} prevalence of 1.5-5% [112,115].

ET gene profile	Isolate #	MLST	CC	*agr* type	*lukSF-I*	sec_{canine}	*se-int*	BacSp222
expA	29-095	765	68	IV	+	-	+	-
	30-085	766	singleton	II	+	-	+	-
	38-056	767	--	III	+	-	+	-
	37-029	768	277	II	+	-	+	-
	41-073	155	54	IV	+	+	+	-
expB	12-005	769	singleton	III	+	-	+	-
	30-090	New1	--	II	+	-	+	-
	31-062	257	258	II	+	-	+	-
	37-024	New2	--	II	+	-	+	-
	39-026	762	singleton	III	+	-	+	-
expA + *expB*	29-002	770	68	II	+	+	+	-
	33-100	771	singleton	IV	+	-	+	-
speta + *siet*	19-040	755	54	IV	+	-	+	-

Table 4: Summary of MLST and selected virulence gene carriage for the 13 *S. pseudintermedius* isolates used for WGS. New 1 and New 2 indicate that the isolates contain novel *ack* gene alleles that were not recorded in the BIGSdb. CC = clonal complex; MLST = multi locus sequence type; -- = CC not able to be determined; singleton = single isolate in CC group; + and - indicate the presence and absence of the indicated gene, respectively.

CHAPTER VI

MOLECULAR CHARACTERIZATION OF S. *AUREUS* ISOLATES FROM HORSES (2007–2017)

Introduction

This study is the first to use WGS methods on a large collection of *S. aureus* isolates, including MRSA and MSSA, collected from horses. WGS has been used to retrospectively investigate and track emerging outbreaks of MRSA in human hospitals and communities [149]. To date, no studies have examined the complete toxin gene carriage profiles of equine MSSA and MRSA strains from equine isolates collected in the southern US, with most studies examining only equine MRSA in Canada or the Northeast and Midwest US [69,70]. The goal of this study was to use WGS to investigate the relationships between strain type, toxin gene carriage and antibiotic resistance in 72 *S. aureus* isolates collected from horses and donkeys that presented to the TAMU VMTH over a ten-year period to investigate the clonality of *S. aureus* isolates cultured from equine patients and to determine if the carriage of toxin genes and antimicrobial resistance genes was similar among isolates.

Since antimicrobial resistance, often to multiple classes of drugs, is a significant problem in community and hospital-acquired strains, we also investigated major genes contributing to antimicrobial resistance in *S. aureus* in the equine isolates. The genes responsible for high-level aminoglycoside resistance in *S. aureus* are *aac (6')-Ie/aph (2'')* (*aac6-aph2*) and *aph (3')-IIIa* (*aph3-III*), and kanamycin/neomycin resistance in

staphylococci is mediated by the plasmid-borne kanamycin nucleotidyltransferase gene, *ant (4')-Ia* or *aadD* [150]. One of the main genes involved in chloramphenicol resistance, *cat*, is carried on the plasmids pC221 and pC223 [151], and trimethoprim-sulfa (TMS) drug resistance is mediated by the *dfr* genes [11]. Efflux pumps encoded by the *tet* genes are major mediators of tetracycline resistance and include the plasmid-borne *tetL* and *tetK* and the transposon-mediated, chromosomally integrated *tetM*; the related multidrug efflux pump encoded by *norA* contributes to fluoroquinolone resistance [11].

While macrolides, such as erythromycin, are not commonly used to treat staphylococcal infections, they are widely used to combat other bacterial infections, and resistance mediated by the *erm*, *msrA*, or *mphC* genes is common in *S. aureus* due to secondary exposure in mixed infections [11]. While rare compared to resistance in other antimicrobial classes, macrolide resistance has been reported in equine-derived *Staphylococcus* spp. isolates [11,151,152], and macrolides are commonly used to treat *Rhodococcus equi* respiratory infections in foals [12]. Treatment with lincosamides, such as clindamycin, is typically contraindicated in horses due to the potentially fatal enterocolitis complications that arise from their use, but resistance (due to the lincosamide nucleotidyltransferase *lnu* genes) is occasionally reported in animal-derived staphylococci [153]. Resistance to spectinomycin and streptomycin is mediated by carriage of s*pc* and *str*, respectively [154]. Mutations in the *rpoB* gene in MRSA strains can confer resistance to rifampicin by altering the ribosomal binding site [11,155]. Fosfomycin is a drug used to treat multi-drug resistant (MDR) strains, but resistance mediated by the *fosB* gene is an emerging problem and is often seen in CC8 isolates from horses [156,157]. Resistance to

quaternary ammonium compound disinfectants, such as chlorhexidine, mediated by *qacA/B, qacC*, or *qacJ* is also relatively common *S. aureus* isolates, particularly of ST1 lineage [158,159]. High-level mupirocin resistance is mediated by transfer of the plasmid carrying *mupA*, a gene that encodes a version of isoleucyl-tRNA synthetase that cannot be bound by mupirocin [11].

In addition to the three classes of toxins discussed in Chapter II, we also investigated carriage of the bacteriophage immune evasion cluster (IEC) genes (*sak*, *scn*, and *chp*), which are often carried with SEA and SEP [160], and the ACME which is thought to mediate colonization of skin in people [117]. The IEC are phage-encoded proteins that integrate into the bacterial genome and function by inhibiting host neutrophil chemotaxis, cleaving host defensing proteins, and inhibiting host opsonization by blocking the formation of C3b on the surface of the bacterium [160].

In summary, we investigated whether carriage of toxin genes, antimicrobial/disinfectant resistance genes, and other virulence factors were associated with ST, *agr* type, or patient clinical outcome (i.e. survival or death) in the collection. We hypothesized that there would be differences in carriage of virulence genes between isolates, and these differences would likely be related to the genetic lineage or clonality of the isolates.

Materials and methods

Collection demographics

The collection consisted of 71 *S. aureus* isolates cultured from 65 horses (*Equus ferus caballus*), six donkeys (*Equus africanus asinus*), and one mule that presented to the Texas A&M University Veterinary Medical Teaching Hospital from July 2007 to January 2017. One environmental *S. aureus* specimen collected in 2008 from a procedure area in the hospital was also included in the study. All patient isolates were residual diagnostic specimens that were collected under the standards of care guidelines for the hospital and with written owner consent obtained to retain isolates for research purposes. Fourteen horse breeds were represented with American Quarter Horse (n = 29), Thoroughbred (n = 7), and American Paint (n = 7) horses being the most common breeds encountered. Of the six donkeys, three were of unspecified breed, two were miniature, and one was an American mammoth donkey. The average age of the equids that contributed isolates in the collection was 8.4 years ± 7.6 years (range 1 day to 31 years). The majority of the isolates were cultured from mares (n = 35) and geldings (n = 25), with fewer isolates from stallions (n = 10) and one isolate recovered from the lung (considered blood/hematogenous isolate) of an aborted male, full-term Clydesdale fetus. Culture site locations were diverse and included skin (n = 26), respiratory secretions or tissues (15), bones or joint tissues (15), blood (6), cornea (5), urine (1), uterus (1), abdominal mass (1), lymph node (1), and environmental (1) sources; the majority of the isolates were collected in 2016 (n =16), 2010 (12), 2014 (12), and 2015 (12) (**Fig. 9**). Bacterial quantification based on growth in the four plate quadrants (i.e. 2+ = growth in 2nd quadrant, etc.) at the time of diagnosis was

available for 43 isolates: 1-6 colonies (n = 8), 1+ growth (8), 2+ growth (13), 3+ growth (9), 4+ growth (5), and isolated only after broth enrichment (7). The urine isolate (33-029) was quantified as 200 cfu/mL. Of the 71 equids, 23 died or were euthanized within 3 weeks of discharge from hospital, with 20 animals receiving a post-mortem examination.

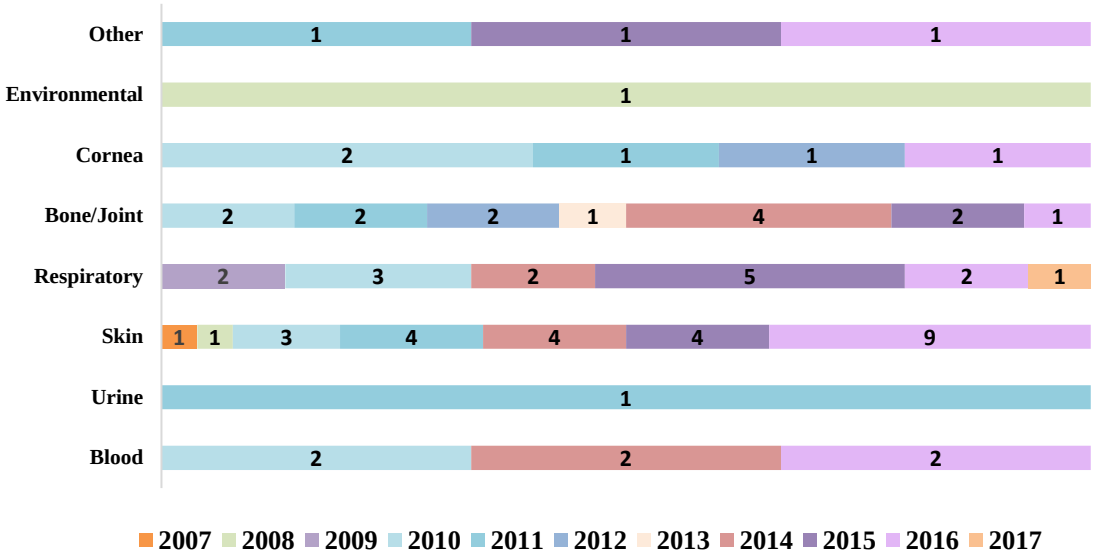

Figure 9: **Distribution of the 72 equine *S. aureus* isolates by culture site and year isolated.** The Other category includes abdominal mass, uterine and lymph node cultures.

Antimicrobial susceptibility and *mecA* testing

Samples from the patients were processed within 24 hours of collection, and the bacteria were speciated as *S. aureus* based on colony morphology and a combination of standard laboratory techniques and/or confirmation with a diagnostic staphylococcal speciation PCR using previously described methods [1,132]. Minimum inhibitory concentration (MIC) data for selected antimicrobials was available for 51 isolates. A commercial assay, using either Vitek (bioMérieux, Durham, NC) or TREK Sensititre

(TREK Diagnostics, Cleveland, OH) MIC plates, was used at time of initial diagnosis to determine antimicrobial susceptibility according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for MIC testing [132]. As isolates were tested for antimicrobial susceptibility as part of patient care, the specific drugs tested varied due to culture site-dependent appropriateness of the antimicrobial panel. Production of *in vitro* β-lactamase was assessed via nitrocefin test in 46 isolates [161]. All 72 isolates were struck onto LB agar containing dissolved mupirocin or vancomycin (Sigma-Aldritch, USA) at a final concentration of 8 µg/mL or 3 µg/mL, respectively, with a 96-pin microplate replicator (Boekel Scientific, Feasterville, PA, USA) and incubated at 37°C for 24 hours for screening of low-level mupirocin and intermediate vancomycin resistance. All isolates were screened via PCR for the presence of the *mecA* cassette at time of initial diagnosis, with *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC ® 43300™ and 29213™ used as *mecA*-positive and -negative control strains, respectively. All isolates that carried *mecA*, regardless of the oxacillin resistance results for isolates with MIC data, were classified as MR for the analyses.

Bacterial culture and DNA extraction

Isolates were preserved at -80°C after initial culture in 20% glycerol-infused lysogeny broth (LB). Isolates were struck onto LB agar and incubated at 37°C for 24 hours. Single colonies for each isolate were inoculated into 1 mL of brain-heart-infusion broth (BD Diagnostics, Franklin Lakes, NJ, USA) and the isolates were incubated at 37°C for 12 hours in an orbital shaker at 200 rpm. The broth cultures were transferred to

microcentrifuge tubes and centrifuged at 8,000 rpm (5,510 x *g*) for 15 min, the supernatant was removed, and the pellets were used for DNA extraction. DNA extraction was performed with a DNeasy® Blood & Tissue Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's recommendations for Gram-positive bacteria, except 1 µl of a solution of 5 mg/mL lysostaphin (L7386; Sigma-Aldritch) derived from *Staphylococcus staphylolyticus* was added to the lysis buffer per isolate.

Library preparation and whole genome sequencing

The aim for the minimum targeted coverage for the combined runs was the same as in Chapter V, approximately 50-fold, and two independent sequencing runs were done for the 72 samples. Sequencing libraries were prepared using the Nextera XT library prep kit (Illumina, San Diego, CA). DNA was quantified using the Qubit™ 2.0 (Life Technologies, Carlsbad, CA) high sensitivity double stranded DNA (dsDNA) assay and all samples were normalized to 0.2 ng/µL. Five microliters of normalized DNA (1 ng total DNA) was used for library preparation. Libraries were prepared on an epMotion® 5075 (Eppendorf, Hauppauge, NY) fluid handling robot adapting the Illumina Library Preparation Protocol for automation. Prepared libraries were checked with the Qubit™ 2.0 dsDNA assay to determine concentration and a TapeStation D1000 HS (Agilent Technologies, Santa Clara, CA) was used to determine the average fragment size of the prepared libraries. All samples were normalized to 4 nM and all 72 samples were pooled together for sequencing. The 4 nM pool was diluted to 20 pM and sequenced on the

Illumina MiSeq 300 x 300 cycle v3 sequencing kit. All run data and FASTQ files were uploaded to BaseSpace (https://basespace.illumina.com/) for downstream analysis.

Genome assembly and annotation

The online sequencing pipeline offered by the Pathosystems Resource Integration Center (PATRIC version 3.4.6; https://www.patricbrc.org) was used to assemble and annotate the *S. aureus* genomes [140]. The Illumina MiSeq paired read libraries for each isolate from the two runs were uploaded to PATRIC and *de novo* assembled using the recommended MiSeq assembly strategy parameter. Briefly, this setting uses Velvet with hash length 35 and trims with BayesHammer. The resulting contigs were then assembled using SPAdes with a k up to 99, and the results were sorted by quality score and combined with Assembly RAST [140]. Genome coverage of the combined MiSeq runs was estimated by comparing the total nucleotide bases per isolate to the 2.82 Mb *S. aureus* subsp. *aureus* NCTC 8325 reference genome in GenBank (NC_007795.1). Annotation was done using the Rapid Annotation using Subsystem Technology tool kit (RASTtk) on the PATRIC pipeline [141].

Strain typing and eBURST analyses

Due to the propensity for alignment errors to occur in tandem repeat areas of the *S. aureus spa* gene with WGS, the *spa* sequences were amplified via PCR as previously described [60] and the forward and reverse strands were Sanger sequenced (Eton Biosciences, San Diego, CA). The *spa* type for each of the isolates was determined using

the tool *spa*Typer (http://spatyper.fortinbras.us/) on the Sanger sequences and compared to the *spa* sequences in the WGS assemblies; new types were submitted to the Ridom SpaServer (http://www.spaserver.ridom.de/) for inclusion in the database. Misalignments of the *spa* gene occurred in 10 of the WGS assemblies, and the *spa* type recorded for the isolates was based on the Sanger sequences. One isolate could not be *dru* typed via WGS and non-amplification of the *dru* was confirmed via the previously described PCR method (ref). Multilocus sequence typing using seven conserved *S. aureus* genes and rMLST using 51 non-paralogous ribosomal genes (excluding allele type for *rpsN* and *rpmG* as recommended for staphylococci) were performed *in silico* with the assembly files as described previously [51,63,76] using the batch query tools on the *S. aureus* MLST (https://pubmlst.org/saureus/) and rMLST (https://pubmlst.org/rmlst/) databases as part of the BIGSdb genomics platform [78].

New MLST *arcC* allele sequences for three isolates were confirmed with Sanger sequencing. New MLST and rMLST allele sequences and types were submitted for inclusion in the respective databases (curator- Keith Jolley). The rMLST alleles for *rpmA*, *rpmF rpsT*, and/or *rpsU* were missing or misaligned in seven of the WGS assemblies, and the respective segments were amplified via PCR with newly designed primers (**Table 5**) and Sanger sequenced (Eton Biosciences) for confirmation of allele type. The missing ribosomal genes were added to the WGS assemblies with Geneious R10 (Biomatters Limited, Auckland, New Zealand) using the consensus sequences for each gene generated from the trimmed forward and reverse Sanger sequenced strands. The MRSA isolates were screened via PCR for amplification of the *dru* segment as previously described [61], and

dru type was assigned *in silico* by querying the *mecA* cassette sequences for each isolate WGS against the online *dru* repeat and typing database (http://dru-typing.org/site/; curator- Richard V. Goering). The SCC*mec* type was assigned via *in silico* BLAST query using the previously described PCR method for typing cassettes I – VI and assigning subtypes [47].

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')	Amplicon size (bp)	Reference
rpmA	AGGGGCTCTTATGCAGTTGT	TATCGTTTGTGCTGGAGCTT	666	This study
rpmF	CGACAAGTTGATCCAAGGCT	TTGGCGGATGAGGTATCCTG	421	This study
rpsT	AAATTTTGATAAGATGAACTCACTTTTAG	AGATGCTTGTGCGGAAACTG	425	This study
rpsU	TTTCACCTCGCCCTCACATT	ACGTTGTTTTAACGGCTTCACA	634	This study

Table 5: PCR primers for amplification of *S. aureus* ribosomal genes for rMLST. PCR reagent concentrations were formulated according to the manufacturer's recommendations (AmpliTaq® DNA polymerase; Thermo-Fisher Scientific). The PCR thermal cycler conditions were initial denaturation at 95 °C for 5 min followed by 30 cycles of 95 °C for 5 min, annealing at 50 °C for 1 min, and extension at 72°C for 1 min, followed by a final extension step of 72°C for 7 min.

Minimum spanning trees were generated using PHYLOVIZ [162], and the full goeBURST plugin was used to cluster *S. aureus* ST in the MLST database (as of September 2017). Founders of CC were assigned based on the ST that had the greatest number of single-locus variants (SLVs; i.e. an isolate that differed from the most STs at only a single locus), and the potential founding isolates of *S. aureus*-associated rMLST groups were also predicted in a similar manner. As of 2014, the historic CC1, CC5, CC8,

CC15, and CC97 are now grouped into one large CC in the MLST database [163], with ST97 as its potential founder. For comparison purposes with other studies, we have kept these CC separate and have retained the conventional founders for each group (i.e. ST1 as founder of CC1, etc.). A phylogenetic tree incorporating 50 (the limit for the method) of the clinical horse isolates, the *S. aureus* subsp. *aureus* NCTC 8325 ST8 reference strain, and outgroups *Macrococcus caseolyticus* JCSC5402, *Staphylococcus saprophyticus* ATCC 15305, and *Staphylococcus epidermidis* ATCC 12228 was constructed with the Phylogenetic Tree Building plugin in PATRIC using the FastTree option and exported to the online Interactive Tree of Life (ITOL v.3; http://itol.embl.de/) interface for editing [140,164,165].

Toxin, antimicrobial resistance, and virulence gene queries

The assembly files and annotated genomes were queried using the standalone BLAST+ suite version 2.6.0 (NIH, Bethesda, MD) and BLAST nucleotide and protein interface in the PATRIC database, respectively. The publically available bacterial antimicrobial resistance gene database Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT) and the staphylococcal VirulenceFinder 1.5 database files available from the Center for Genomic Epidemiology (both current as of March 2017) were used to make the database for the BLAST+ queries [145,166]. Nucleotide sequences in GenBank for *lukPQ* (LT671578.1), *qacA/B* (GU565967.1), *qacC* (M37889.1), phenol soluble modulins 1-4 (BK006301.1) and *chp* (AF285146.1) were also added to the database. A positive hit for a gene was defined as a query having at least 96 % identity with and covering at least 90 % of the length of the database gene reference. The *agr* type was assigned based on

BLAST query using primer sequences from the previously described PCR typing method [80]. The IEC type, based on the presence of the *sea* or *sep* and *scn, sak,* or *chp* genes, was assigned *in silico* via BLAST query using the previously described PCR typing scheme [160]: A = *sea*+*sak*+*chp*+*scn*, B = *sak*+*chp*+*scn*; C = *chp*+*scn*, D = *sea*+*sak*+*scn*, E = *sak*+*scn*, F = *sep*+*sak*+*chp*+*scn*, G = *sep*+*sak*+*scn*. To ensure that all the toxin and virulence genes were detected in the genomes, the raw MiSeq FASTQ files were converted to FASTA files with fastq2fasta (TM Software, Inc., Arcadia, CA) and searched with a combination of the PCR primer sequences specific for each enterotoxin [167] or IEC [160] gene and the previously mentioned BLAST+ VirulenceFinder database to account for sequence variation. Potential hits were then queried against the NCBI database using the online BLAST-n interface; a cut-off of > 90% identity to a known toxin or virulence gene sequence cataloged in GenBank was used to identify the genes. Potential regions containing prophages were analyzed with PHAST [142].

Results and discussion

Assembly findings

The average coverage achieved over the combined MiSeq runs for all isolates was 64-fold (range, 41 to 90-fold), with removal of one outlier (60-073) that had 174-fold coverage. Nine isolates had coverage estimates lower than the benchmark goal of ≥ 50-fold. While overall genome coverage was high, coverage of some areas containing highly repetitive regions, such as the *spa* gene and the region flanking the *egc* and *seh*, was low in 10 isolates, resulting in fragmentation of genes. In these instances, either Sanger

sequencing or conversion and BLAST query of the reads for each isolate (as outlined in the methods) was performed to accurately assess type and gene carriage.

Clinical and post-mortem findings

The most common clinical findings reported for the equids were subcutaneous abscesses/cellulitis or dermatitis (n = 17), osteomyelitis and/or joint infections (15), pleuropneumonia (6), and corneal ulcers (5). In the dermatitis cases, most of the lesions were located on the limbs (n = 10). Two horses had documented recent travel history from Canada (Horse 33) and France (Horse 64). Two horses were cultured (penile and clitoral fossa swabs) as part of a breeding soundness exam. Nine of the cases involved infection of surgical sites, two involving infection of joint implants and seven involving skin infections related to incisional dehiscence. Cytologic examination of fluid or tissue aspirates was performed by a clinical pathologist in 22 of the cases involving joint (n = 8), eye (5), respiratory (5), abdominal (2), skin (1), and urinary (1) infections. Neutrophilic to suppurative inflammation was observed in the tissues of 19 cases and, within that subset, coccoid bacteria were seen in 10 cases. In the majority of the cases ending in death or euthanasia (65%; 15/23), the reason could be attributed to bacterial infections in which *S. aureus* was cultured. Of the 20 animals receiving a full necropsy, the gross findings included bronchopneumonia (n = 6; **Fig. 10A**), chronic joint infections (5), wounds or skin abscesses (5), corneal ulcers (2), a nasal mass (1), and abdominal abscesses (1; **Fig. 10B**). In one case (Horse 68), death resulted from severe, secondary bacterial infection at sites of previous erythema multiforme (**Fig. 10C**). Histologic findings included

necrosuppurative bronchopneumonia with Gram-positive cocci (**Fig. 10D** and inset), synovitis and tenosynovitis, and subcutaneous or intraabdominal abscesses.

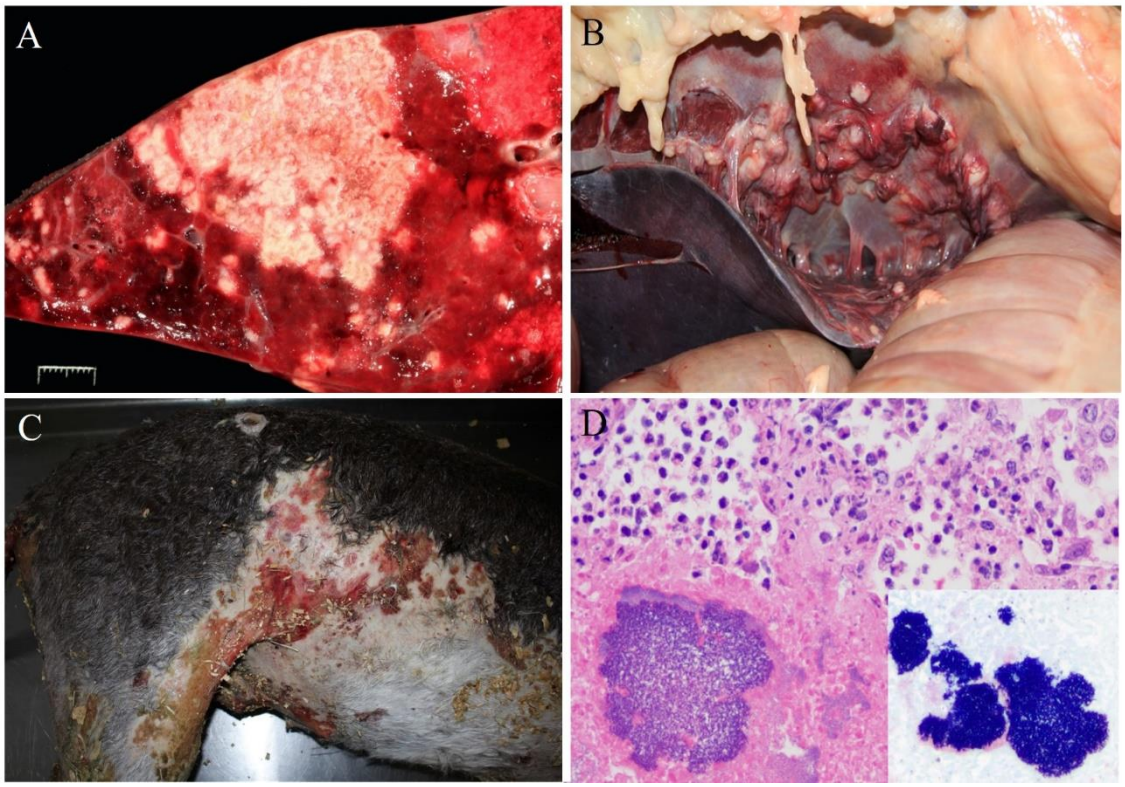

Figure 10: Gross and histologic lesions attributed to *S. aureus* infection in horses.
A- Horse 33, sectioned lung, necrosuppurative bronchopneumonia.
B- Horse 52, diaphragm and liver, suppurative peritonitis and hepatitis with adhesions.
C- Donkey 68, flank, multifocal ulcerative and suppurative dermatitis.
D- Horse 33, lung, necrosuppurative pneumonia with large clusters of bacteria (H&E); inset- Gram-positive cocci in lesion (Hucker-Twort Gram stain).

Typing characteristics

Eighteen distinct ST were represented, with ST1 (n = 18), ST133 (11), ST8 (9), and ST97 (9) most frequently encountered. Three new MLST (4214, 4215, and 4277) were identified due to single point mutations in the *arcC* gene. The most common *spa* types were t127 (n = 13), t1294 (6), t064 (6), and t008 (5). Two new *spa* repeats were discovered r667 (AAAGAAGATCGCAACAAGCCTGGT) and r748 (AAAGGAGACAACAAAAAACCTGGC); six new *spa* types were discovered in MS isolates: t17107 (repeats 03-16-21-149-23-13-17-149-23-24), t17108 (03-16-21-17-23-13-17), t17109 (03-16-21-17-23-13-17-17-17-23-667), t17110 (03-13-17-17-23-24), t17118 (15-16-34-34-33-13), and t17146 (15-12-16-748-16-02-25-17-24-24), representing one isolate each. The ST and *spa* type combinations observed are illustrated in **Fig. 11**. The most common ST- *spa* type pairings were ST1- t127 (n =13), ST816- t1294 (6), ST8- t008 (5), ST97- t2112 (4), and ST8- t064 (4). The two horses with documented travel outside the US cultured ST1-t127 MSSA, a major ST circulating in human communities in Canada and the northern US [136] and one of the most predominate equine MSSA strains in Denmark [37]. The overall prevalence of MSSA in the collection was 81%, and the majority of the MSSA isolates were ST1-t127 (n =13), ST816-t1294 (6), ST97-t2112 (4), or ST133-t1166 (3). ST1, ST133, and ST97 lineages are commonly cultured from livestock in Canada, Europe, and Africa [37,96,120,168,169]. The *spa* type t1294 has been described in one study of equine isolates in Denmark [170], but it has not been linked to ST816 or been described in the US before. The isolates cultured from the

lesions in the equids illustrated previously had the following typing characteristics: Horse 33- ST1-t127-57195; Horse 52- ST4277-t701-57185; Donkey 68- ST133-t2420-54226.

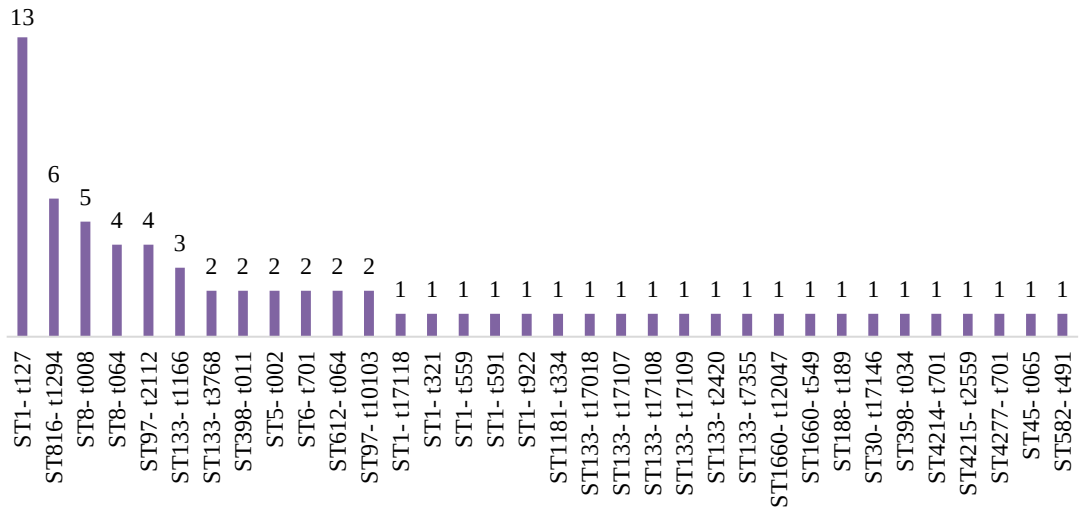

Figure 11: Distribution of ST and *spa* type pairings for the 72 equine *S. aureus* isolates.

The overall prevalence of MRSA (defined as *mecA*-positive isolates) in our study was 19%. Of the 14 *mecA*-positive isolates, there were 64% ST8 (9/14), 14% ST612 (2/14), 14% ST398 (2/14), and 7% ST5 (1/14). SCC*mec* types observed included IVa (n = 7), IVd (6), and IIa (1). Four *dru* types were identified: dt9g (n = 5), dt10a (4), dt10q (2), and dt7d (2). The ST5 isolate (38-086) was untypable because it contained a deletion in the *dru* region that was confirmed by absence of a *dru* amplicon via the conventional PCR method. There were two distinct groups of ST8 MRSA: five isolates characterized by SCC*mec* type IVa, *spa* type t008, *dru* type dt9g, and four isolates having SCC*mec* IVd, *spa* t064, and *dru* dt10a. Most of the equine MRSA cases in our study were attributable to

the USA 300 clone (ST8-IV-t008; PVL-positive) [117], a clone implicated in the majority of outbreaks of CA-MRSA in the US, and the Canadian USA500 clone (ST8-IV-t064; PVL-negative) [169]. The high percentage of ST8-associated MRSA is similar to cases in veterinary hospitals in the northeastern US, Ohio and Canada attributable to USA500 and to infections at French stud farms [34,69,70,169]. However, in contrast to findings in European horses [34,120,171] where the originally porcine-associated ST398 is a predominant clone, only two of the MRSA isolates in our study were ST398. Methicillin-resistant strains were collected from two of the horses that died, but only one was infected with a MR strain (51-045) that directly contributed to death. Of the nine surgical site infections, three were attributed to MR strains: a tarsal incision line infection caused by a ST8-IVd-t008-dt9g isolate, a celiotomy incisional infection caused by a ST612-IVd-t064-dt7d isolate, and a paralumbar fossa incisional infection caused by a ST398-IVa-t011-dt10q isolate. A full minimum spanning tree based on MLST was drawn for the *mecA* positive and negative isolates (**Fig. 12**) using the goeBURST algorithm in PHYLOViZ, and a summary of the findings for the 14 *mecA*-positive isolates is given in **Table 6** .

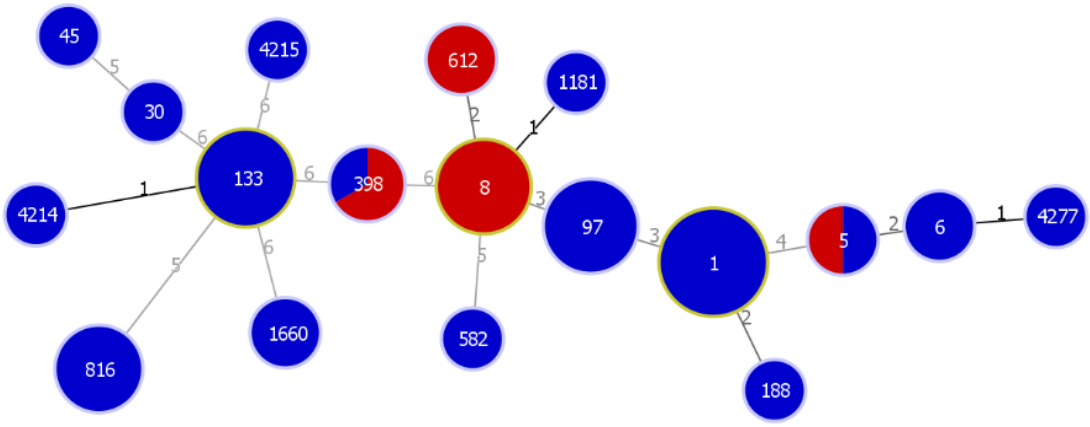

Figure 12: Full minimum spanning tree for the 72 equine *S. aureus* isolates. The red shading indicates the proportion of MRSP isolates for the indicated ST and the blue indicates MSSP. The size of the spheres reflects the number of isolates of that type, and the numbers on the connecting lines indicate % similarity in increments of 0.5% (i.e. 1 = 99 to 99.5% similarity; 2 = 98.5 to 99% similarity, etc.).

Equid ID	Isolate ID	Isolation Year	Age (yrs)	Sex	Breed	Infection site	Outcome	MLST	CC	SCC*mec* type	*spa* type	*dru* type	rMLST
Enviro	11-028	2008	--	--	--	--	--	8	8	IVd	t064	dt10a	53899
2	11-038	2008	14	Mare	Quarter Horse	Skin	Survived	8	8	IVd	t064	dt10a	53899
6	20-093	2010	18	Gelding	Quarter Horse	Respiratory	Survived	8	8	IVa	t008	dt9g	4320
15	29-021	2010	30	Gelding	Mixed Breed	Respiratory	Survived	8	8	IVa	t008	dt9g	4320
16	29-031	2010	4	Mare	Quarter Horse	Cornea	Died	8	8	IVa	t008	dt9g	57201
19	31-073	2011	10	Gelding	Mixed Breed	Skin[a]	Survived	8	8	IVa	t008	dt9g	4320
27	38-086	2012	14	Gelding	Quarter Horse	Cornea	Survived	5	5	IIa	t002	NA	4326
28	40-086	2012	6	Mare	Quarter Horse	Bone/Joint	Survived	8	8	IVa	t008	dt9g	4320
37	49-069	2014	0.2	Mare	Donkey	Bone/Joint	Survived	398	398	IVa	t011	dt10q	4340
41	51-045	2014	0.08	Stallion	Donkey	Respiratory	Died	8	8	IVd	t064	dt10a	53899
42	51-058	2014	6	Gelding	American Paint	Skin	Survived	8	8	IVd	t064	dt10a	54220
46	56-089	2015	16	Mare	Quarter Horse	Skin[a]	Survived	398	398	IVa	t011	dt10q	57692
51	60-070[*]	2015	31	Mare	Arabian	Respiratory	Survived	612	8	IVd	t064	dt7d	54222
53	61-017	2015	3	Gelding	Thoroughbred	Skin[a]	Survived	612	8	IVd	t064	dt7d	54222

Table 6: Clinical and strain typing characteristics of the 14 *mecA*-positive *S. aureus* isolates.
*phenotypically methicillin-sensitive isolate; [a]surgical site infection; CC = clonal complex; Enviro = environmental isolate; NA = not amplifiable due to *dru* deletion event.

Nine CC were represented and groupings are represented in the phylogenetic tree (**Fig. 13**): CC1 (n = 19), CC133 (12), CC8 (12), CC97 (9), CC5 (5), CC398 (3), CC15 (1), CC45 (1), and CC30 (1). Two ST (1660 and 4215) could not be assigned to CC, and ST816 was a singleton. All of the CC8 isolates were MRSA, and CC8 is one of the most common complexes associated with MRSA in horses [157]. The CC133 and CC97 groups are typically associated with livestock [96,104], and CC15 has rarely been cultured from donkeys in Tunisia [96]. The CC5 (USA100), CC30 (USA200), and CC45 (USA600) strains are typically associated with human infections and are the most common CC contributing to blood infections and endocarditis in people [53]. Mucosal infections in people are predominately caused by CC30, and CC45 is a frequent colonizer of human skin in the US and northern Europe [53]. Forty-nine rMLST types were observed, and 41 new types were identified in this study. The most common rMLST were 4390 (n = 8), 4320 (4), 54212 (4), 57189 (4), and 53899 (3). The 4390 (ST1), 4320 (ST8), 6128 (ST816), and 4340 (ST398) types were identical to the rMLST of European strains deposited in BIGSdb. The new rMLST 57189 (ST816), 53899 (ST8), 54212 (ST97), 54222 (ST612), and 57200 (ST133) occurred in multiple isolates in the collection and may represent substrains that developed in the US.

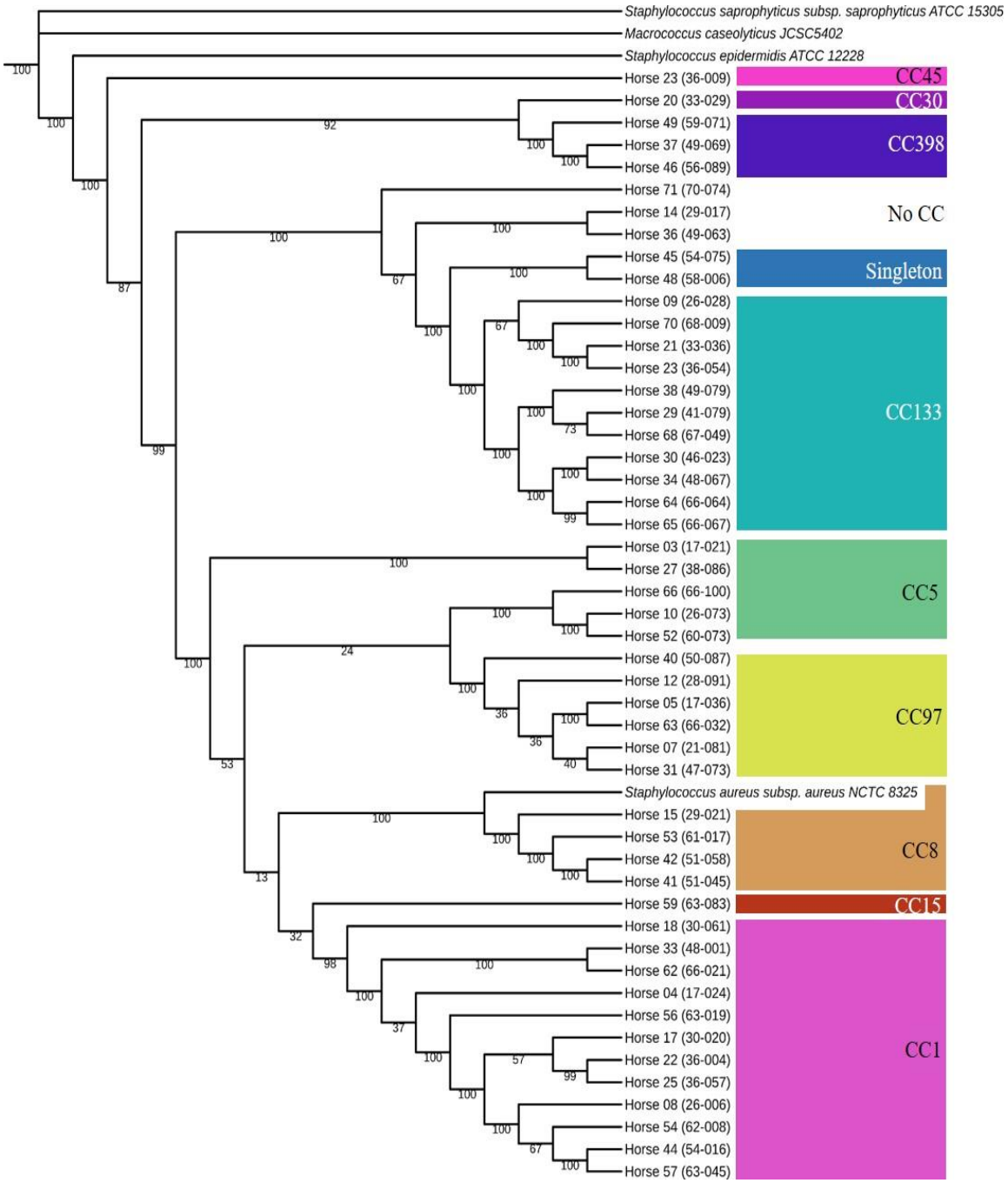

Figure 13: Phylogenetic tree of 50 of the equine *S. aureus* clinical specimens. The associated CC are labeled at right. The numbers on the nodes are the bootstrap values. Branch lengths have been ignored.

Toxin and virulence gene carriage

Exfoliative and membrane damaging toxins

None of the isolates carried ETA, ETB, or ETD. Alpha-hemolysin was carried by 92% (66/72) of the isolates, and β-hemolysin was present in 97% (70/72) of the isolates. All isolates carried aureolysin and the γ-hemolysin HlgA, HlgB, and HlgC components. None of the isolates carried *lukMF'* or any of the EDIN genes. Panton-Valentine leucocidin was only found in the five ST8-IVa-t008-dt9g isolates, USA300, which is a human CA-MRSA strain [104]. The majority of the isolates carried *lukPQ* (66%; 48/73) and carriage was associated with presence of the strain 3711 prophage (99% identity to reference LT671578.1). Interestingly, all of the *lukPQ*-positive isolates were MSSA, and the CC traditionally associated with human infections, such as CC8, 15, and 30 [53], were *lukPQ*-negative. While most of the *lukPQ*-positive isolates also harbored *lukED* (81%; 58/72), none of the PVL-positive isolates also carried *lukPQ*. This is the first report of *lukPQ* carriage in *S. aureus* isolates from the US. In the paper first describing *lukPQ* [91], the genes were found in 15% of 87 isolates tested from the Netherlands, Italy, and Portugal, and demonstrated *lukPQ* carriage in ST1, ST133, ST398, and ST1660 isolates from horses. However, *lukPQ* was not found in a 26-isolate MRSA subset used in a previous study from The Ohio State University [69], and the ST816, ST6, and ST97 have not been described to carry *lukPQ*. The authors did not mention the methicillin resistance status of the isolates they examined in the study, but as only MRSA was reported in the Ohio study, most of the isolates used in the initial *lukPQ* prevalence study were likely also MRSA. Given that we did not observe any *lukPQ*-positive MRSA isolates in our

collection, the higher prevalence of *lukPQ* in our study with US isolates is likely due to also screening MSSA or potential differences amongst US vs. European MRSA *S. aureus* isolates. Carriage of *lukAB* was also common (66%; 48/72), but all of the *lukAB*-positive isolates in the collection also carried *lukPQ.* None of the isolates carried the four phenol soluble modulin genes; however, one isolate (66-100) carried a gene with 97% identity to *psmA3*.

Superantigens

Many of the isolates carried enterotoxin or enterotoxin-like genes, including *sea* (n = 8), *seb* (4), *sec2* variant (6), *sed* (1), *tsst-1* (7), *seg* (9), *seh* (18), *sei* (11), *sej* (1), *sel-k* (4), *sel-l* (6), *sel-m* (11), *sel-n* (11), *sel-o* (11), *sel-p* (1), *sel-q* (4), *ser* (1), *sel-u* (11), and *sel-x* (68). Carriage of multiple enterotoxins and SEl in a single isolate was common, with 96% (69/72) of isolates carrying at least one enterotoxin or SEl gene. None of the ST398 isolates carried enterotoxin genes, a finding that is similar to other prevalence reports in Europe that evaluated enterotoxin carriage [120]. *Sel-x* was the most common enterotoxin gene encountered, occurring in 94% of the isolates, and the ubiquitous carriage in our study is similar to previous reports [106]. Enterotoxin H was carried by all of the ST1 isolates and was not observed in other types. Previous studies have reported 100% prevalence of *seh* in ST1 isolates [96,104]. Some of the CC8 MRSA isolates carried *sea, seb, sel-k, sel-q,* and *sel-x*. The K+Q+X enterotoxin combination is one of the most common reported for USA300/CC8 isolates [53]. While most isolates carried two or fewer toxins genes, the six isolates belonging to ST816-t1294 and one ST5-t002 isolate carried

10 toxin genes. The toxin combinations in relation to ST are illustrated in **Fig. 14**. All of the ST816 isolates carried *sea*, *sec2, sel-l, tsst-1* and the *egc2* (composed of *seg, sei, sel-m, sel-n, sel-o,* and *sel-u*), and the *tsst-1* amino acid sequences were identical. The region containing *tsst-1* was different in the ST816 isolates when compared to the ST1 *tsst-1*-positive isolate (57-034). In ST816, *tsst-1* was carried on a prophage with *sel-l* and two genes similar to *sec2*, whereas carriage of these genes was not observed in 57-034. An intact prophage sequence was found in ST816 isolate 58-006 via PHAST analysis that had 94% identity to the *S. aureus* Φ282 prophage (KT809368.1), but covered only 51% of this reference sequence. The *tsst-1* sequence in 58-006 had 95% identity to the *S. aureus* N315 reference sequence (NC_007795.1) and 97% identity to the 57-034 *tsst-1*. The ST816 prophage likely carries a *tsst-1* variant that is common in ST816 isolates. The CC5 MRSA isolate (38-086) carried a plasmid encoding *sed* and *ser* in addition to *egc2* as is commonly observed in CC5/USA100 isolates [53,104].

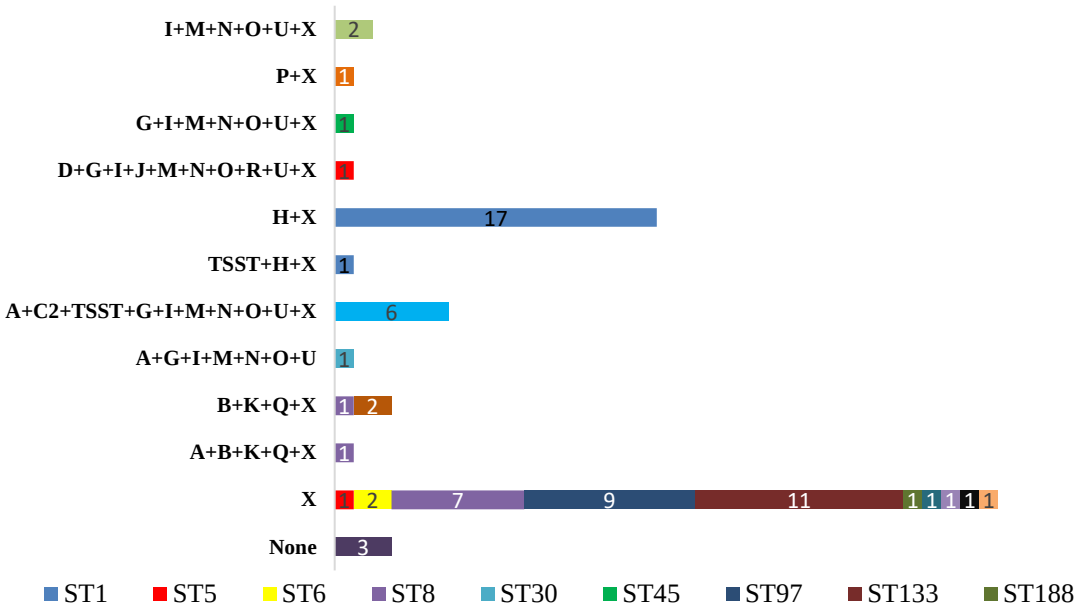

Figure 14: Enterotoxin and enterotoxin-like gene carriage combinations vs. ST.

Other virulence genes

The majority of the isolates had *agr* type I (57%; 41/72) or III (26%; 19/72) systems with fewer having type II (15%; 11/72) or IV (1%; 1/72). In a study of *S. aureus* isolates collected from donkeys in Tunisia, the most common *agr* represented were also types I and III [96]. All of the ST8 isolates carried the ACME. Eleven of the isolates carried IEC genes with the following IEC and ST pairings observed: A in ST30 (n =1); B in ST8 (5), ST5 (2), ST398 (2), ST188 (1), and ST45 (1); C in ST582 (1); D in ST8 (1); and G in ST1181 (1). Types B and D are the most common IEC types encountered in isolates with type I *agr* systems, and type A is commonly found in isolates with type III *agr* systems [160]. The lack of the *sak* gene in the ST582/CC15 isolate is similar to previous reports in this lineage [104].

Antimicrobial MIC and resistance gene carriage

MIC data

The majority (68%; 49/72) of the isolates were tested via commercial MIC plate assay at time of diagnosis against panels of commonly used antimicrobial drugs. Antimicrobial drugs tested included: gentamicin (n = 49 isolates tested), ampicillin (47), ceftiofur (47), chloramphenicol (47), enrofloxacin (47), tetracycline (47), trimethoprim/sulfamethoxazole (TMS; 47), penicillin (46), oxacillin (45), amikacin (36), doxycycline (36), cefazolin (34), rifampin (34), ticarcillin with clavulanic acid (34), ceftazidime (33), and erythromycin (30). The sensitivities for selected antimicrobials are given in **Fig. 15**. Of the 49 isolates with plate MIC data, most were resistant to the β-lactam drugs ampicillin (51%; 24/47), and penicillin (48%; 22/46) due to *in vitro* production of β-lactamase. Fifteen of the isolates were designated as resistant to β-lactams based on production of β-lactamase in the nitrocefin test. Intermediate or full resistance to ceftiofur (45%; 21/47), tetracycline (34%; 16/47), and gentamicin (31%; 15/49) was also prevalent. Resistance to rifampin (12%; 4/34), enrofloxacin (7/47; 15%), chloramphenicol (19%; 9/47), and doxycycline (19%; 7/36) was uncommon. The 96-pin plate assays for intermediate vancomycin and low-grade mupirocin resistance revealed seven intermediately (4 μg/mL) vancomycin resistant isolates and one mupirocin resistant isolate (17-021) in the collection. The high level of resistance to β-lactam drugs and tetraclycine in the isolates was similar to previous reports in *S. aureus* from horses in Canada and Europe [34,172]. However, the percentage of gentamicin, TMS, and rifampin

resistant isolates in the collection is higher than the zero resistance prevalence observed in one report from Canada and lower than reports from French stud farms [34,172].

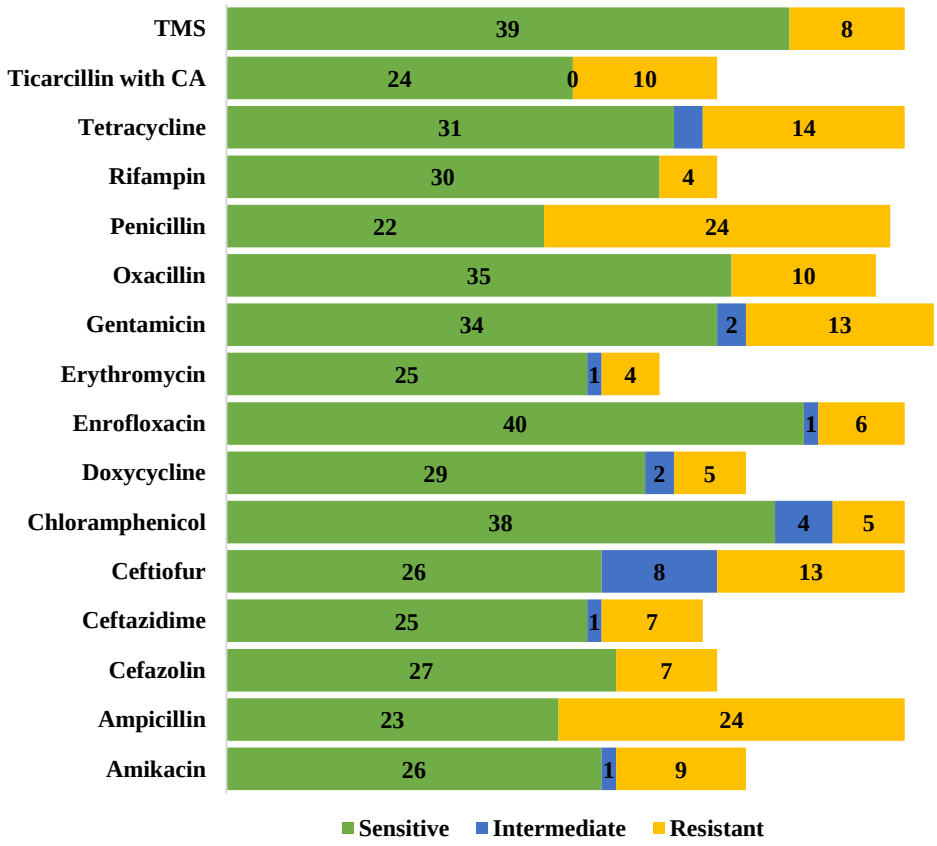

Figure 15: **Antimicrobial susceptibilities for selected drugs for 49 *S. aureus* isolates as determined through commercial MIC plate assay**. CA = clavulanic acid; TMS = trimethoprim/sulfamethoxazole.

Antimicrobial resistance gene carriage

The antimicrobial resistance genes carried by the equine isolates were diverse. The β-lactamase encoding *blaZ* gene was present in 27 isolates (38%), including 11 of the 14 *mecA*-positive isolates. Of the 24 penicillin-resistant isolates identified in the MIC plate

assay or via nitrocefin test, 15 carried *blaZ*. One isolate (17-036) carried *blaZ* but was sensitive to penicillin, and one resistant isolate (17-021) did not carry *blaZ.* The majority (15/16) of the resistant isolates with penicillin MIC data (**Fig. 16**) carried *blaZ*, but eight isolates deemed resistant by the nitrocefin test did not carry *blaZ.* This discrepancy is most likely due to false-positives on the nitrocefin test, which have been reported with animal-derived *S. aureus* isolates [161]. Distribution of MR and MS isolates between culture sites was similar (**Fig. 17**). None of the isolates carried high-level vancomycin resistance genes (i.e. *vanA*). Nine of the 10 MRSA isolates evaluated by MIC plate assay were phenotypically resistant to oxacillin and carried *mecA*, while one isolate (60-070) was phenotypically oxacillin sensitive but was *mecA*-positive. This phenotypically oxacillin sensitive isolate was a ST612-IVd-t064-dt7d-54222 and had identical typing characteristics to the MRSA isolate 61-017 from Horse 51 that was being treated for a surgical site infection. Although one report has indicated a plasmid carrying a non-functional version of the β-lactamase regulatory gene BlaR1 is responsible for *mecA* repression in SCC*mec* type IV isolates (which have non-functional *mecA*-regulatory genes) [173], no deletion in BlaR1 similar to that described in the report could be found in 60-070. However when the BlaR1 sequence in 60-70 was compared MRSA 61-017, 60-70 did have an Arg-23-Lys substitution near the N-terminus in a similar area to the reported deletion. The significance of this mutation on BlaR1 functionality in 60-070 would require further study.

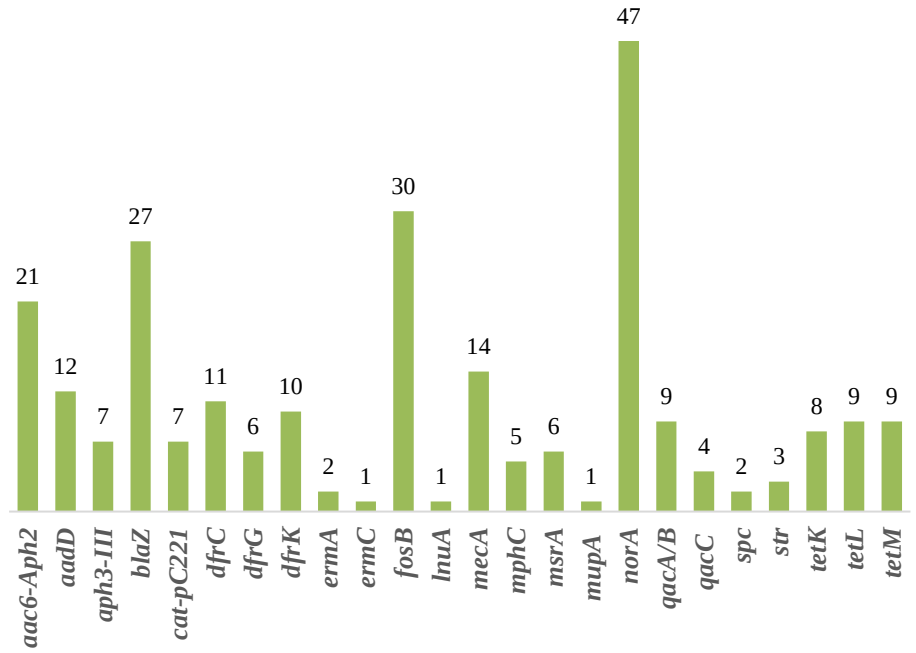

Figure 16: Antimicrobial resistance genes carried in the equine *S. aureus* isolates.

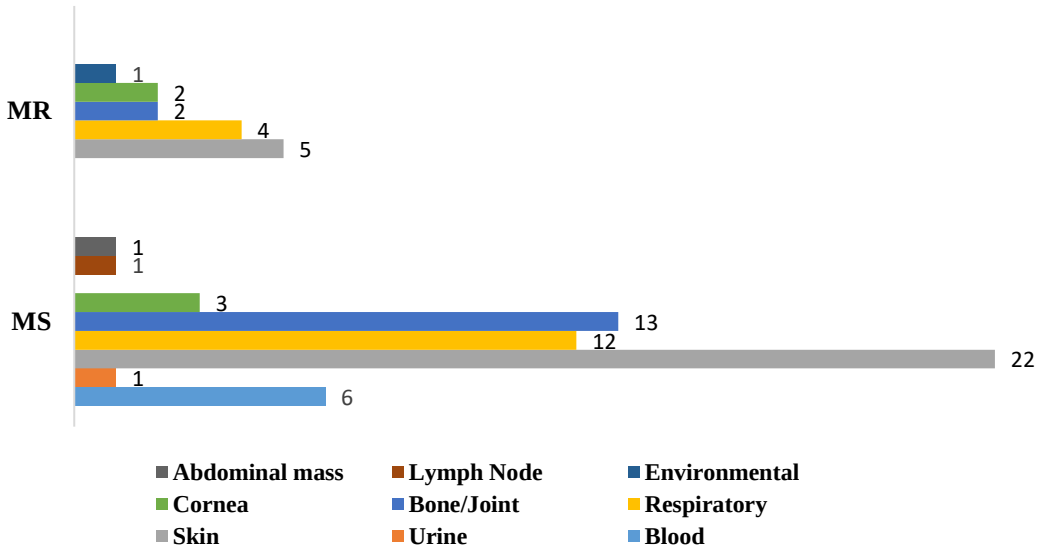

Figure 17: **Distribution of the 72 *S. aureus* isolates by *mecA* positivity and culture site**. MR = *mecA*-positive, MS = *mecA*-negative.

Aminoglycoside resistance genes were commonly encountered (39%; 28/72) and included: single carriage of *aac6-aph2* (n = 9), dual carriage of *aac6-aph2* and *aph3-III* (2), single carriage of *aph3-III* (5), single carriage of *aadD* (2), and dual carriage of *aadD* and *aac6-aph2* (10). All of the gentamicin intermediately resistant and -resistant isolates identified on MIC plate assay carried aminoglycoside resistance genes. The single carriage *aadD* isolates were ST5, which is prevalent in CC5 isolates [104]. The aminoglycoside resistance genes were carried mostly by CC1, CC5, CC8, CC15, CC97, and CC398 isolates. This is similar to the distribution reported for human ST1 [159] and livestock ST398 [154] isolates in Ireland, porcine ST97 isolates in Italy, and CC8 and CC5 human isolates worldwide [104]. The only chloramphenicol resistance gene encountered in the collection was chloramphenicol acetyltransferase encoded on the pC221 plasmid (n = 7) carried by ST8 (n = 3), ST1 (1), ST1660 (1), ST816 (1), and ST612 (1) isolates, which is not commonly reported in these ST; all of the chloramphenicol-resistant isolates determined by MIC carried the pC221 plasmid.

The tetracycline resistance genes *tetK*, *tetL*, and *tetM* were detected in eight, nine, and nine isolates, respectively; dual carriage was also observed: *tetK* and *tetL* (n = 1) and *tetK* and *tetM* (1). Presence of the *norA*-encoded drug efflux pump was common (65%; 47/72). Based in the MIC data, the TMS resistance gene, *dfrK*, was found only in phenotypically TMS-sensitive isolates. The TMS resistance genes were commonly (38%; 27/72) observed: *dfrC* (n = 11; ST1, ST8, and ST612), *dfrG* (6; ST1, ST97, ST133, ST398, and ST1660), and *dfrK* (10; ST1 and ST398). Animal-derived *S. aureus* isolates frequently carry *dfrK* [154]. The *dfrK*-encoded protein sequence in the isolates which also carried

tetL had only 96% identity with the *dfrK* reference sequence in the ARG-ANNOT database, suggesting that there may be functional differences between the proteins. Carriage of *tetL* was linked with *dfrK* in the ST1-t127 isolates due to carriage on the same plasmid, but Tn559-mediated single carriage of *dfrK* was also observed in the two ST398-MRSA-IVa isolates as has been previously described in porcine isolates from Germany [174]. In one isolate, 26-006, the *dfrK* gene was missing from the *tetL* plasmid, most likely due to a plasmid recombination event, but the isolate also carried *dfrC* which conferred high-level TMS resistance.

Macrolide resistance genes were observed in ten isolates (14%): *ermA* (n = 2; ST5), *ermC* (1; ST8), *msrA* (1; ST582), or dual carriage of *msrA* and *mphC* (5; ST8 or ST97). All of the erythromycin-resistant isolates identified on MIC plate assay carried resistance genes. Isolate 17-036 carried *msrA* and *mphC* but was phenotypically erythromycin-sensitive; there were no mutations in *msrA* or *mphC* in 17-036. Equine CC8 isolates have been reported to carry *ermC* [157,169], and dual carriage of *msrC* and *mphC* has been reported in human SCC*mec* type IV isolates in Japan [123]. One isolate (17-021) cultured from pleural fluid in Horse 3 carried the *lnuA* gene imparting lincosamide resistance and also carried the *mupA* high-level mupirocin resistance plasmid; this case represents the first report of these genes in an equine-derived isolate. Interestingly, none of the horses that cultured macrolide-resistant isolates were being treated with a macrolide antimicrobial drug or mupirocin while in the hospital and use of clindamycin or other lincosamides is contraindicated in horses.

Carriage of spectinomycin (*spc*) and streptomycin (*str*) resistance genes was rare with two ST5 isolates carrying *spc* and three ST8-PVL+ isolates carrying *str*. Thirty isolates (42%) carried plasmids encoding the fosfomycin-resistance gene, *fosB*, with 40% (12/30) of these isolates also carrying *mecA*. All of the CC5, CC8, CC15, CC30, and CC133 isolates in the collection carried *fosB*, and the gene was not carried by isolates in the other observed CC. This *fosB* prevalence rate is similar to previous reports with CC5, CC30, and CC133 isolates of human and animal origin [156,175]. The four rifampin resistant isolates identified by MIC plate assay were all MR and did have mutations in the *rpoB* gene associated with phenotypic resistance in *S. aureus* or *E. coli* [155]: Asp-471-Val, Asp-471-Asn, and His-471-Asn; with an additional three isolates identified in the collection with identical mutations that would most likely confer rifampin resistance. Nine isolates (13%) carried the quaternary ammonium compound resistance genes *qacA/B* (all ST1-t127) and four isolates (6%) carried *qacC* (ST5, ST8, and ST1660). Carriage of *qacA/B* genes is prevalent in ST1-t127 isolates [159] and *qacC* has been rarely detected in porcine ST97 isolates [168].

Conclusions

The overall prevalence of MRSA in our collection was 19%, and one ST612/CC8 *mecA*-positive but phenotypically oxacillin sensitive isolate was found in the collection. The predominant MRSA clones were the CA-MRSA USA 300 clone (ST8-IV-t008; PVL-positive) and the Canadian USA500 clone (ST8-IV-t064; PVL-negative), with most of the isolates coming from skin and respiratory tissues. The most common MSSA CC were

ST1, CC133, and CC97, and the most common ST-*spa* type was ST1-t127. New *spa* types (n = 6), MLST (3), and rMLST (41) were discovered in this study. No ET genes were identified in the WGS of the equine isolates. The novel purported equine adapted leucocidin, LukPQ, was found in 66% of the isolates, and all of the positive isolates were MSSA. This is the first report of *lukPQ* carriage in *S. aureus* isolates cultured outside of Europe. Carriage of *sel-x* was most common, followed by *seh+sel-x* in the ST1 isolates. All of the ST816 isolates carried a prophage that encoded *sea*, *sec2*, *sel-l* and a novel form of *tsst-1*. Carriage of the IEC was occasionally observed (11%) with type B being most common. Most of the isolates were resistant to β-lactam antimicrobials, and resistance to ceftiofur (45%; 21/47), tetracycline (34%; 16/47), and gentamicin (31%; 15/49) was also prevalent. The antimicrobial resistance genes *norA, fosB, blaZ, aac6-aph2* and *aadD* were commonly carried by the isolates. The quaternary ammonium compound resistance genes *qacA/B* and *qacC* were carried by 18% of the isolates and carriage was most common in ST1-t127 isolates. One ST5 isolate (17-021) collected from the pleural fluid of an American Paint horse stallion was mupirocin-resistant (*mupA*) and carried the lincosamide resistance gene *lnuA*, the first report of these resistance genes in an equine-derived isolate. The association of MSSA with the majority of the equine infections, most cultured in significant amounts with cytologic or histopathologic confirmation, indicates that MSSA can be pathogenic in horses. The diverse array of toxin and antimicrobial resistance genes found in the equine MSSA isolates also could contribute to patient morbidity, and the MSSA isolates could serve as reservoirs for transfer of toxin or resistance genes between staphylococci of similar lineage.

CONCLUSIONS

Staphylococcus spp. are important causes of skin and systemic infections in people, dogs, and horses. Methicillin-resistant S. *aureus* and MRSP infections are common in humans and animals. In dogs, *S. pseudintermedius* is the most common cause of pyoderma, and MRSP is a significant contributor to soft tissue infections in dogs. Horses colonized with *S. aureus* can be asymptomatic carriers, but methicillin sensitive and MRSA infections have been associated with significant morbidity and mortality in horses and bacteria can be transferred between humans and horses. *Staphylococcus aureus* ET genes are associated with blistering skin lesions, and four purported exfoliative toxin genes have been reported in *S. pseudintermedius*: *speta, siet, expA,* and *expB.* Few studies have determined the prevalence of *S. pseudintermedius* ET genes in a large collection of canine isolates, and it is unknown what conditions facilitate native induction of SIET, EXPA, or EXPB, or if dogs produce antibodies to *S. pseudintermedius* ET. There has also been limited study of MSSA infections in horses and previous studies have not examined the complete toxin gene carriage profiles of equine MSSA and MRSA strains collected in the southern US. There were four aims of this study, and the findings are summarized below.

AIM 1: Determine the prevalence of exfoliative toxin (ET) gene carriage in *Staphylococcus pseudintermedius* isolated from dogs. Determine if ET gene carriage is correlated to inflammation severity in canine skin biopsies. The prevalence of *speta, siet, expA*, and *expB* in a collection of 500 clinical isolates from healthy and diseased dogs

was 100, 99, 13, and 16%, respectively. The observed ET gene combinations were *speta* alone (0.6%; 3/500), *speta+siet* (71%; 357/500), *speta+siet+expA* (12%; 60/500), *speta+siet+expB* (15%; 73/500), and *speta+siet+expA+expB* (1.4%; 7/500). The *expA* and *expB* genes were always carried concurrently with *siet*. There were 159 methicillin-resistant (MR) *S. pseudintermedius* isolates; 147 in the diseased dog group and 12 in the healthy dog group. The overall MRSP prevalence in the collection was 32%, and the most common SCC*mec*, *spa*, and *dru* type combinations were V+t06+dt11a. There was no correlation between methicillin resistance status or specific typing characteristics and carriage of *expA* or *expB.* A higher proportion of the dogs with pyoderma cultured *expA* -positive isolates when compared to healthy and diseased dogs. The inflammation severity scores of the 25 paired biopsies were correlated to the ET carriage profile of the associated bacterial isolates (P = 0.0002; FET). The *speta+siet* profile was less likely to be associated with mild inflammation (P = 0.0001; two-tailed FET), and *speta+siet+expA* and *speta+siet+expB* were more likely to be associated with mild inflammation (P = 0.0333 and 0.0162, respectively; two-tailed FET) when compared to other severity categories. However, the inflammation scoring study should be repeated with a larger sample size, preferably with bacterial culture directly from the collected skin biopsies, to determine if there were biases in our collection.

AIM 2: Induce native expression of the EXPA and EXPB toxin proteins in bacterial isolates *in vitro*. Determine if dogs with and without a history of pyoderma or culture of *S. pseudintermedius* produce antibodies to the ET *expA* and *expB*. Native

induction of EXPA and EXPB was achieved thorough incubation of liquid cultures with 10% CO_2, and both proteins were excreted in the culture supernatant. Expression of EXPB and EXPA was observed for the single and dual carriage isolates with bands that corresponded to the predicted sizes for each of the proteins. SIET was only labeled in the synthetic peptide controls on nitrocellulose dot blot and in the recombinant SIET+GFP protein, and native expression of SIET was not observed in the tested *S. pseudintermedius* samples. Sera from the pyoderma/exposed and control dog groups contained antibodies to purified EXPA and EXPB, with antibodies in the pyoderma/exposed group also binding to a band of the approximate size of the recombinant SIET+GFP from Set 1 as well as multiple proteins in the *E. coli* bacterial lysate.

AIM 3: Perform whole genome sequencing (Illumina MiSeq and Oxford Nanopore) on select *Staphylococcus pseudintermedius* isolates carrying specific ET genes to map the positions of the ET genes within the chromosome and determine if ET genes are integrated on mobile genetic elements (MGE). Whole genome sequencing was performed on a subset of 13 *S. pseudintermedius* isolates. In the eight isolates that carried *expA*, the ET gene was consistently carried adjacent to a reverse transcriptase (Group II intron) that is purported to facilitate horizontal transfer of genes in other bacterial species. The *expB* gene was also adjacent to mobile element proteins that may facilitate horizontal transfer of the gene, but the region upstream to *expB* was variable between the examined isolates. The MLST, *agr*, and *spa* types were different amongst the 13 sequenced isolates. Additional sequencing is needed to bridge the gap in the *expA*

region to determine if the upstream and downstream are similar among the isolates. Further study is needed to determine if the regions flanking the *expB* gene influence expression of the toxin, and to test whether *expA* and *expB* can be horizontally transferred between isolates of a similar genetic background (i.e. strain type).

AIM 4: Perform whole genome sequencing (Illumina MiSeq) on *Staphylococcus aureus* isolates from horses admitted to the Veterinary Medical Teaching Hospital. Determine the prevalence of toxin gene carriage in the isolates and if there are correlations between toxin gene carriage, patient demographics, infection site, or methicillin resistance status. The MRSA prevalence in the collection of 71 clinical isolates from horses and donkeys was 19%, with most being clonal complex 8. The predominant MRSA clones were the CA-MRSA USA 300 clone (ST8-IV-t008; PVL-positive) and the Canadian USA500 clone (ST8-IV-t064; PVL-negative), with most of the isolates coming from skin and respiratory tissues. The most common MSSA CC were ST1, CC133, and CC97, and the most common ST-*spa* type was ST1-t127. New *S. aureus spa* types (n = 6), MLST (3), and rMLST (41) were discovered in this study. No ET genes were identified, and the ST816 isolates carried a prophage encoded variant of toxic shock syndrome toxin not previously characterized in isolates from horses. Further characterization of the ST816 TSST-1 carrying prophage is needed to determine if the variant is equine-adapted and produces similar effects to previously described TSST-1 variants in an animal model. The novel purported equine adapted leucocidin, LukPQ, was found in 66% of all isolates, the first instance of carriage outside of Europe. Carriage of

lukPQ only occurred in the MSSA isolates in our collection. The antimicrobial resistance genes *norA, fosB, blaZ, aac6-aph2* and *aadD* were commonly carried by the isolates. The quaternary ammonium compound resistance genes *qacA/B* and *qacC* were carried by 18% of the isolates and carriage was most common in ST1-t127 isolates. One ST5 isolate (17-021) was mupirocin-resistant (*mupA*) and carried the lincosamide resistance gene *lnuA*, the first report of these resistance genes in an equine-derived isolate. We found that MSSA in addition to MRSA carry diverse virulence factors and can be pathogenic in horses.

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